OES NOT CIRCULATE

The Journal of LABORATORY and CLINICAL MEDICINE

UNIVERSITY OF MICHIGAN MAR 12 1957 MEDICAL LIBRARY

VOL. 49 MARCH, 1957 NO. 3

The Official Publication of the Central Society for Clinical Research

Clinical and Experimental

In Vivo and In Vitro Metabolism of Insulin-I^{131} and Glucagon-I^{131} in Normal and Cortisone-Treated Rabbits. Solomon A. Berson, M.D., Rosalyn S. Yalow, Ph.D., and Bruno W. Volk, M.D., Bronx, N. Y. ---------- 331

The Simultaneous Occurrence of Rheumatoid Arthritis and Aggammaglobulinemia. Robert A. Good, M.D., Jerome Rötstein, M.D., and William F. Mazzitello, M.D., Minneapolis and St. Paul, Minn. ---------- 343

A Serologic Investigation of Waldenström's Macroglobulinemia by Means of the Agar Diffusion Method. F. Westendorp Boerma, Ph.D., and E. Mandema, M.D., Groningen, The Netherlands ---------- 358

Serum Protein Fractions in Lymphogranuloma Venereum. Ninan Verghese, B.Sc., M.B., B.S., M.Sc., M. P. Satyanarayana, B.Sc., P. N. Ramakrishnan, B.Sc., B. Pharm., and K. Kuppuswamy, M.Sc., Madras, India ---------- 370

Proteolytic Activity of Whole Human Serum Following Surgery. Wayne M. Meyers, Ph.D., Kenneth L. Burdon, Ph.D., and Maxine N. Riley, B. A., Houston, Tex. 377

Serum Protein Turnover in Multiple Myeloma. Solomon A. Berson, M.D., and Rosalyn S. Yalow, Ph.D., Bronx, N. Y. ---------- 386

The Diuretic Activity of a New Pyrimidinedione Administered Orally to Nonedematous Adults. Mervin L. Clark, M.D., and James A. Hagans, M.D., Oklahoma City, Okla. ---------- 395

A Study of the Excretion of Rb^{86}, K^{42} and K^{39} in Thermal Sweating. G. S. Berenson, M.D., and G. E. Burch, M.D., New Orleans, La. ---------- 401

Absorption and Excretion of Sulfamethoxypyridazine: A New, Long-Acting Antibacterial Sulfonamide. Roger L. Nichols, M.D., and Maxwell Finland, M.D., Boston, Mass. 410

Serum Vitamin B_{12} Level and Binding Substance of Tuberculous Patients With and Without Liver Disease. Robert L. Davis, ScD., Robert C. Duvall, M.D., and Bacon F. Chow, Ph.D., Baltimore, Md. ---------- 422

The Effects of Sodium Lactate on Renal Metabolism in Patients With Kidney Disease. Archer P. Crosley, Jr., M.D., John F. Brown, M.D., Benjamin Schuster, M.D., Dean A. Emanuel, M.D., Herman Tuchman, M.D., Cesar Castillo, M.D., and George G. Rowe, M.D., Madison, Wis. ---------- 429

(Contents continued on inside front cover)

PUBLISHED BY THE C. V. MOSBY COMPANY, ST. LOUIS 3, MISSOURI

Copyright © 1957 by The C. V. Mosby Company

Contents—*(Continued from front cover)*

Studies on Anti-V: A Recently Discovered Rh Antibody. Eloise R. Giblett, M.D., Jeanne Chase, B.S., and Arno G. Motulsky, M.D., Seattle, Wash. ---------- 433

The Persistence in the Blood of the Radioactive Label of Albumins, Gamma Globulins, and Globulins of Intermediate Mobility. IV. Specific Activity Ratios Between Plasma and Edema Fluid of I^{131}-Labeled Albumin Injected Intravenously in Nutritional and Nephrotic Hypoproteinemia. D. S. Kushner, M.D., D. Bronsky, M.D., A. Dubin, M.S., B. P. Maduros, M.D., and S. H. Armstrong, Jr., M.D., Chicago, Ill. ---------- 440

Laboratory Methods

A Colorimetric Method for Transaminase in Serum of Plasma. W. W. Umbreit, Ph.D., Rahway, N. J., G. R. Kingsley, M.S., R. R. Schaffert, A.B., Los Angeles, Calif., and Herman Siplet, A.B., Philadelphia, Pa. ---------- 454

The Relationship Between Bacteriophages of Salmonellae and Their O Antigens. John H. Silliker, Ph.D., and Welton I. Taylor, Ph.D., Chicago, Ill. ---------- 460

Photoelectric Method for Estimation of the Oxygen Saturation of Nonhemolyzed Whole Blood. Paul Sekelj, D.Eng., and Arnold Johnson, M.D., Montreal, Quebec ----- 465

Isolation of Serum Lipoproteins by Zone Electrophoresis. Joseph Bernsohn, Ph.D., Hines, Ill. ---------- 478

A Micromethod for the Determination of Salicylic Acid in Plasma. Sing-Pao Chiang, Ph.D., and Smith Freeman, M.D., Ph.D., Chicago, Ill. ---------- 481

A Titration Method for the Determination of Calcium in Serum Using a New Indicator. Marie A. Andersch, Ph.D., Baltimore, Md. ---------- 486

A Method for Concentrating and Determining Iodide in Urine. Harold L. Helwig, Ph.D., William A. Reilly, M.D., and James N. Castle, A.B., San Francisco, Calif. 490

Simplified Technique for Determining Serial Changes in Plasma Volume Using I^{131} Human Serum Albumin. Bernard Abrams, M.D., Tilden C. Everson, M.D., Ph.D., Theodore Fields, M.S., and Ervin Kaplan, M.D., Chicago, Ill. ---------- 494

(Editorial and Business Communication on inside back cover)

Editor
WILLIAM D. ROBINSON, M.D.

Associate Editor
WILLIAM H. BEIERWALTES, M.D.

University Hospital
University of Michigan Medical School
1313 Ann Street, Ann Arbor, Michigan

Board of Editors

S. HOWARD ARMSTRONG, JR., M.D.
University of Illinois, Chicago

FRANK H. BETHELL, M.D.
University of Michigan, Ann Arbor

RICHARD B. CAPPS, M.D.
Northwestern University Medical School, Chicago

A. C. CORCORAN, M.D.
Cleveland Clinic, Cleveland

ELMER L. DeGOWIN, M.D.
State University of Iowa, Iowa City

RICHARD V. EBERT, M.D.
University of Arkansas, Little Rock

WENDELL H. HALL, M.D.
University of Minnesota, Minneapolis

RICHARD LOUIS LANDAU, M.D.
University of Chicago, Chicago

OSCAR D. RATNOFF, M.D.
University Hospitals, Cleveland

W. A. SODEMAN, M.D.
University of Missouri, Columbia

RANDALL G. SPRAGUE, M.D.
Mayo Clinic, Rochester

RICHARD W. VILTER, M.D.
University of Cincinnati, Cincinnati

Vol. 49, No. 3, March, 1957. The Journal of Laboratory and Clinical Medicine is published monthly by The C. V. Mosby Company, 3207 Washington Blvd., St. Louis 3, Mo. Subscription rates: United States and its Possessions $13.00, Students $6.50; Canada, Latin-America, and Spain $13.50, Students $7.00; Other Countries $14.50, Students $8.00. Single copies $2.00 postpaid. Entered as Second Class Matter October 8, 1915, at the Post Office at St. Louis, Missouri, under Act of March 3, 1879. Printed in the U. S. A.

The Journal of Laboratory and Clinical Medicine

VOL. 49 MARCH, 1957 No. 3

CLINICAL AND EXPERIMENTAL

IN VIVO AND IN VITRO METABOLISM OF INSULIN-I^{131} AND GLUCAGON-I^{131} IN NORMAL AND CORTISONE-TREATED RABBITS

SOLOMON A. BERSON, M.D., ROSALYN S. YALOW, PH.D., AND BRUNO W. VOLK, M.D.
BRONX, N. Y.

IT HAS been reported recently[1] that pretreatment of rabbits with large doses of cortisone regularly produces an increase in the fasting blood sugar and exaggerates the initial hyperglycemic effect of commercial regular insulin. Prolonged hyperglycemia lasting as long as three hours usually followed the intravenous administration of 500 units insulin to cortisone-treated rabbits. The duration and extent of hyperglycemia were correspondingly lessened by reduction of insulin dosage but differences between normal and cortisone-treated rabbits were still observed at the 10 unit dosage level. "Glucagon free" insulin produced an initial hyperglycemia in cortisone-treated rabbits with doses of 200 units or greater but not with smaller doses.

In addition to the aforementioned apparent hypersensitivity of cortisone-treated rabbits to the glucagon contained in commercial insulin preparations,[1] a decrease in sensitivity to exogenous insulin in "steroid diabetes" has been suggested.[2, 3] Therefore, it seemed of interest to compare the rates of metabolism of both insulin and glucagon labeled with I^{131} in normal and cortisone-treated rabbits.

From the Radioisotope Service of the Veterans Administration Hospital, Bronx, N. Y., and the Isaac Albert Research Institute of the Jewish Chronic Disease Hospital, Brooklyn, N. Y.

Received for publication Aug. 6, 1956.

METHODS

Crystalline regular insulin* and crystalline glucagon* were trace-labeled with I^{131} at a level of less than 1 iodine atom per molecule of protein according to methods described previously.[4] Although bio-assay of the labeled insulin showed no detectable loss of biologic potency, it has been shown[5] that such assays may not disclose as small a degree of alteration as may be induced by irradiation from the I^{131}. Since some of the altered labeled products bind to the plasma proteins and thus persist in the circulation much longer than does unaltered insulin-I^{131}, the presence of even a small fraction of altered material may affect significantly the later course of disappearance of protein-bound I^{131} from the blood.[5] Therefore, in all experiments reported here, irradiation damage to the labeled hormones was minimized by the addition of serum albumin[6] after separation of unbound I^{131} by rapid dialysis, and, in addition, studies in cortisone-treated and control rabbits were performed simultaneously with each lot of the labeled hormones. Analysis by paper electrophoresis[5] showed that all lots of insulin-I^{131} employed in the present studies contained less than 1 to 2 per cent of altered material.

While glucagon was found to be much less sensitive than insulin to the effects of irradiation,[7] it appeared to be more labile in other respects, with as much as 5 to 10 per cent alteration frequently observed on analysis by paper electrophoresis. Although iodination and dialysis at a pH of 9.5 to 10.0 appeared to minimize the extent of these alterations, several lots were unsatisfactory and had to be discarded. Even though precise bio-assays with glucagon-I^{131} were not carried out, the experiments to be reported indicated, at least grossly, the maintenance of considerable potency in inducing hyperglycemia at a dosage level of about 2 μg per kilogram.

Cortisone-treated rabbits were given daily intramuscular injections of 50 mg. cortisone acetate in saline suspension (Upjohn) for 7 to 14 days up to and including the day preceding the experiments. All studies were performed in rabbits which had fasted for approximately 18 hours. Following administration of insulin-I^{131} or glucagon-I^{131} into an ear vein, heparinized blood samples were taken from a vein of the opposite ear at various intervals. Whole blood was analyzed for glucose according to the Nelson modification of the Somogyi micromethod.[8] Radioactivity in whole plasma and in the washed precipitates of plasma treated with cold 10 per cent trichloracetic acid was assayed in a well scintillation counter. Paper strip electrophoretograms of plasma were also scanned for radioactivity. The methods of I^{131} assay and of paper electrophoresis have been described previously.[5] In several cases butanol extracts of the plasma were chromatographed on paper in a n-butanol-acetic acid-water system with the butanol phase as the solvent.[9]

Liver homogenates were prepared by mincing liver with 2 volumes of 0.067 M phosphate buffer in a Virtis homogenizer. After centrifugation at 2,000 g for 5 minutes, the supernatant extract was diluted 1:10 and incubated at 37° C. with insulin-I^{131} or glucagon-I^{131} in phosphate buffer at pH 7.4 to 7.6. The rate of degradation of the labeled hormones was followed by serial assays of TCA-precipitable and nonprecipitable radioactivity. In some instances, further enzymatic degradation after a certain interval was inhibited by the addition of 25 to 50 mg. 1-butyl-3-p-tolylsulfonylurea sodium (Orinase)† and the I^{131}-labeled substances were analyzed by paper electrophoresis and chromatography.

RESULTS

In Vivo.—In order to compensate for differences in the sizes of the rabbits, all concentrations are plotted as body $wt._{Kg.} \times$ fraction of the dose administered/liter plasma. Figs. 1 and 2 depict the time course of disappearance of protein-precipitable radioactivity from the plasma of insulin-I^{131} and glucagon-I^{131},

*We are indebted to Drs. O. K. Behrens and C. W. Pettinga, of Eli Lilly and Company, for a generous supply of crystalline regular insulin lot No. 535664 and to Dr. Behrens for a supply of crystalline glucagon.

†We are indebted to Dr. Cornelius O'Donovan of The Upjohn Co., Kalamazoo, Mich., for a generous supply of Orinase.

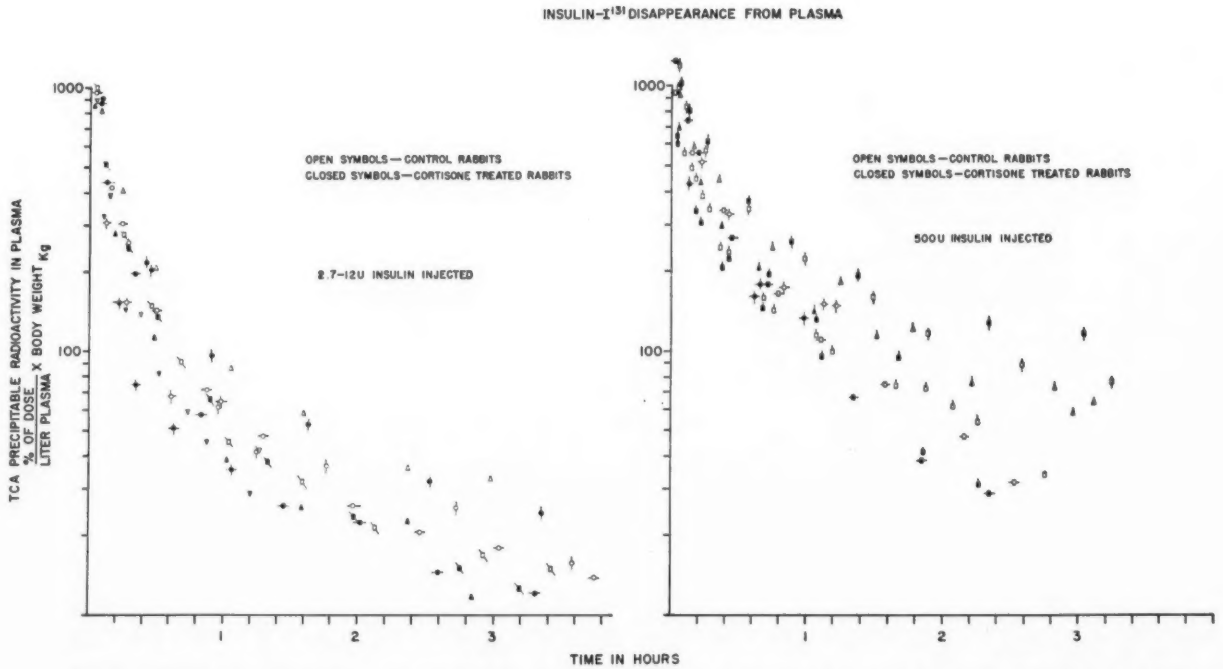


Fig. 1.—Plasma concentrations of precipitable radioactivity as a function of time in cortisone-treated and control rabbits following the intravenous administration of I^{131}-labeled insulin accompanied by small doses (left) and large doses of unlabeled insulin (right). Like symbols indicate that the same preparation of insulin-I^{131} was employed.

respectively, in cortisone-treated and control rabbits. Not shown is the accompanying increase in nonprecipitable radioactivity. The curves fail to reveal any detectable differences between cortisone-treated rabbits and control rabbits. For small doses of both insulin-I^{131} and glucagon-I^{131}, precipitable radioactivity in the plasma for the later points represents, primarily, damaged material bound to serum proteins rather than the unaltered I^{131}-labeled hormones, which comprise the bulk of the administered radioactivity. Paper radioelectrophoretograms, containing only the protein-bound radioactivity (after heat coagulation,

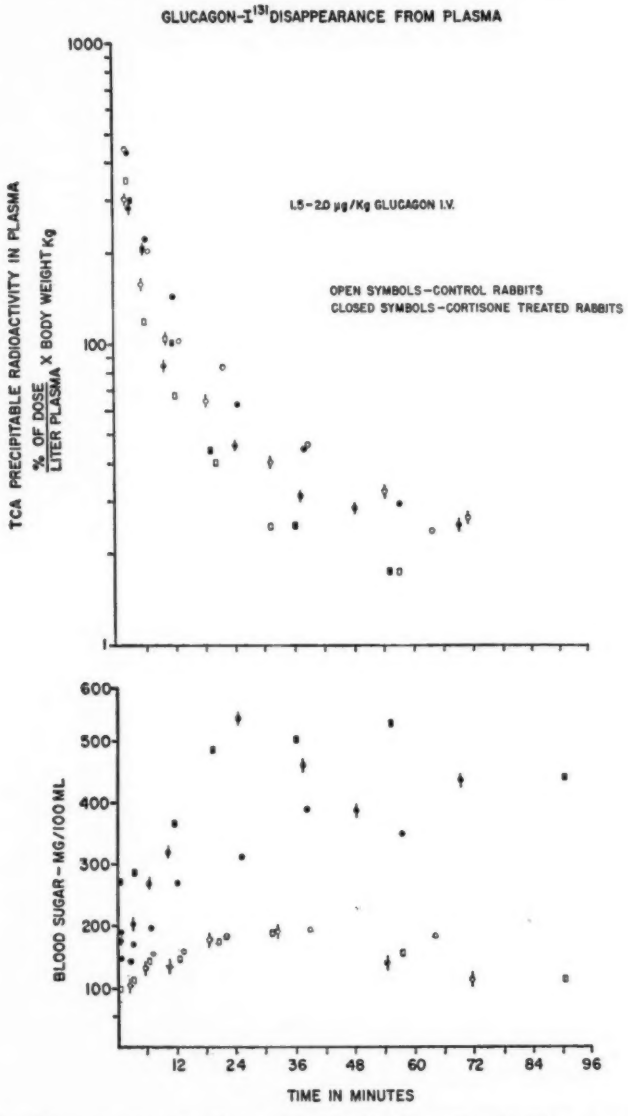


Fig. 2.—Plasma concentrations of precipitable radioactivities as a function of time in treated and control rabbits following the intravenous administration of I^{131}-labeled glucagon. Like symbols indicate that the same preparation of glucagon-I^{131} was employed. Note the high-fasting blood sugar levels and the marked hyperglycemia following glucagon in the cortisone-treated rabbits.

staining, and washing) of plasma samples taken from a rabbit after administration of glucagon-I^{131}, illustrate this point (Fig. 3). Glucagon-I^{131} itself is adsorbed to the paper at the site of application and comprises the main fraction of protein-bound radioactivity shortly after administration; only a very small amount is altered material which binds to the serum proteins. With time, however, glucagon is removed rapidly from the circulation and the altered material bound to the plasma proteins becomes a progressively larger fraction of

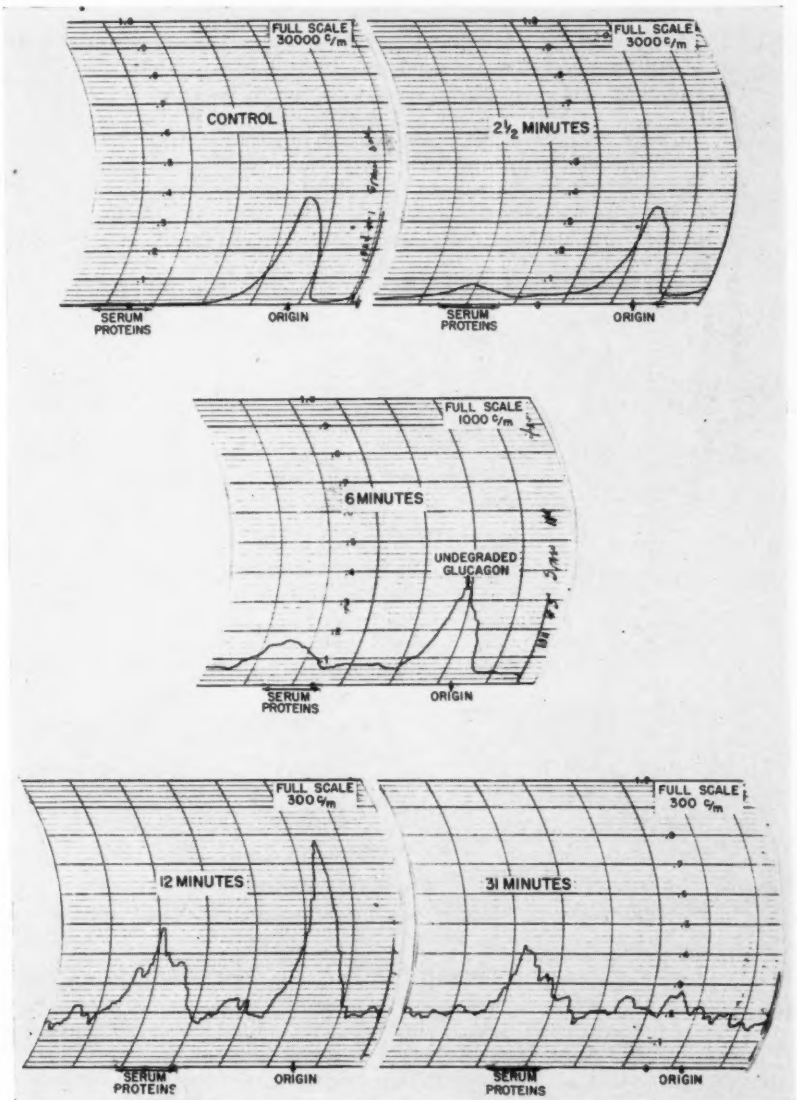


Fig. 3.—Short-run (one hour) paper electrophoresis combined with hydrodynamic flow of serial plasma samples following administration of glucagon-I^{131}. Line of application ("origin") near cathodal vessel. Undegraded glucagon is adsorbed at origin and water flow effects movement of nonadsorbed radioactivity toward anode. Paper strips stained and washed after heat coagulation of protein. Only protein-bound activity remains. Compare with same strips before staining and washing (Fig. 5).

the total protein-bound I^{131}. Similar observations with insulin-I^{131} have been described previously.[5] Since the amount of altered material may vary somewhat from lot to lot of the labeled hormones, a somewhat greater variability in the values for protein-bound radioactivity of the *late* plasma points (Figs. 1 and 2) is not unexpected.

Fig. 1 also shows the slower rate of removal of insulin-I^{131} when the labeled hormone is accompanied by enormous doses (500 units) of stable insulin. In Fig. 4 this effect is shown again by comparing in the same animals the disappearance of the same preparation of insulin-I^{131} when given alone and when accompanied by 500 units of stable insulin.

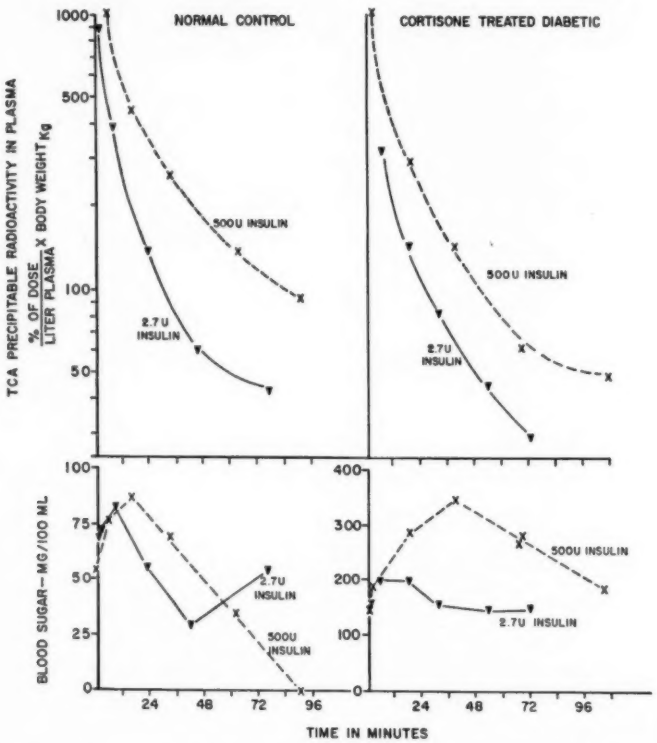


Fig. 4.—Plasma concentrations of precipitable radioactivity (above) and blood sugar response (below) of cortisone-treated (right) and control rabbits (left) following intravenous administration of 2.7 units and 500 units insulin accompanied by tracer doses of insulin-I^{131}.

That the disappearance of I^{131} from the TCA-precipitable fraction and its appearance in the nonprotein-bound fraction of plasma is not attributable simply to deiodination of the labeled hormones, but is a manifestation of peptide bond hydrolysis, has been demonstrated by analysis of paper electrophoretograms and chromatograms. Fig. 5 depicts the changes in distribution of total radioactivity on paper electrophoretograms as a function of time following intravenous administration of glucagon-I^{131}. Since the strips were scanned for radioactivity prior to staining and washing, both protein-bound

and protein-free I^{131} are recorded. Electrophoresis combined with hydrodynamic flow[5] having proceeded for about one and one-half hours, the following radioactive fractions could be distinguished by comparison with the stained strips: (1) unaltered glucagon-I^{131} or insulin-I^{131}, (2) iodide^{131}, (3) I^{131}-labeled altered fractions bound to and precipitable with serum proteins, (4) other labeled fractions not bound to plasma proteins and soluble in methanol or water solutions after heat coagulation of proteins on the paper. Accompanying the

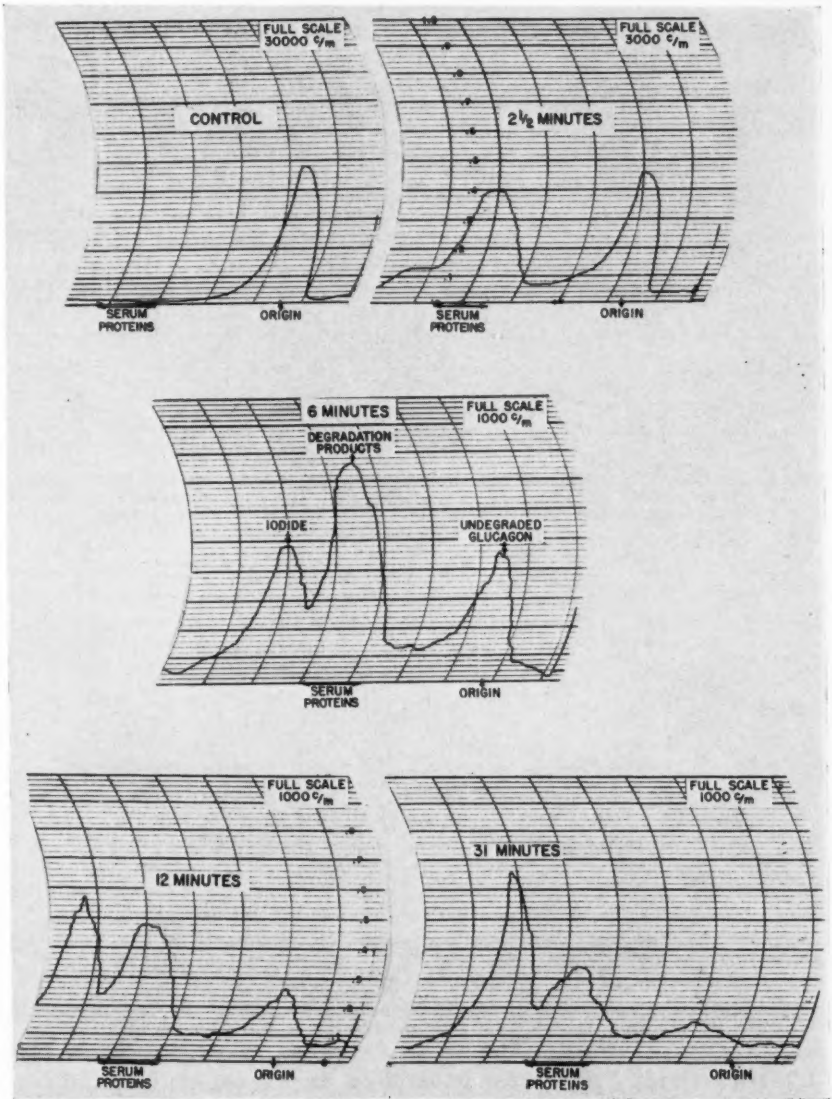


Fig. 5.—Same paper strips as in Fig. 3 before staining and washing. In addition to undegraded glucagon and plasma protein-bound radioactivity, nonprotein-bound labeled degradation products (fraction 4 of text) and iodide^{131} are present. The latter two fractions are removed during staining and washing procedures.

disappearance of glucagon-I^{131} in this experiment is the appearance of fraction 4 which is followed by the appearance of progressively increasing amounts of iodide^{131}. The altered fractions bound to the serum proteins are present in the initially administered material and show only a slow gradual decrease in concentration in the plasma (Fig. 3). The appearance of fraction 4 prior to the appearance of significant amounts of iodide and the increase in concentration of iodide concentration concomitant with the fall in concentration of fraction 4 indicate the latter as the precursor of iodide. When butanol extracts of the plasma were chromatographed on paper, this fraction was observed to migrate with an R_f value identical to that of monoiodotyrosine* (Fig. 6).

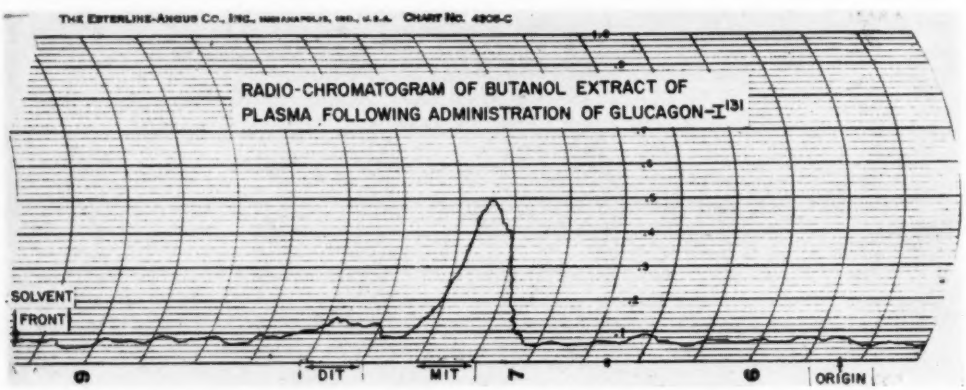


Fig. 6.—Radiochromatogram of butanol extract of two-and-one-half-minute specimen of Fig. 5. Monoiodotyrosine (MIT) and diiodotyrosine (DIT) were added to this specimen prior to chromatography. The strip was stained with ninhydrin after drying.

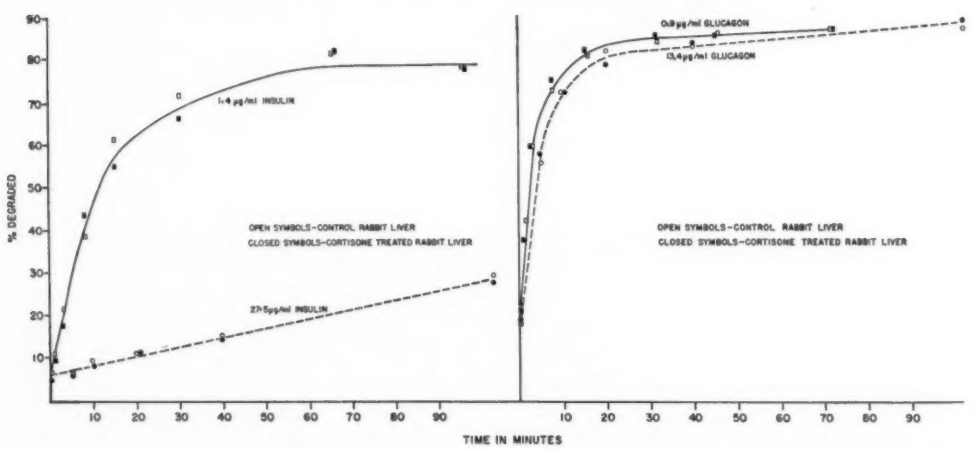


Fig. 7.—Degradation of insulin-I^{131} (left) and glucagon-I^{131} (right) by homogenates of cortisone-treated and control rabbit livers as a function of time.

In Vitro.—Studies with homogenates of liver from control and cortisone-treated rabbits also failed to reveal any significant differences in the rate of

*At the level of iodination employed with these hormones, it is to be expected that monoiodotyrosine would comprise the major fraction of iodinated amino acids produced in the protein.

metabolic degradation of insulin-I^{131} or glucagon-I^{131} at tracer levels or at load levels (Fig. 7). The limited supplies of crystalline glucagon available did not permit studies with more highly concentrated glucagon solutions, but the very high glucagonase* activity present and the limited solubility of glucagon at a physiologic pH make it appear that the V_{max} for glucagon degradation can only be obtained at much lower concentrations of liver enzyme than employed here.

Paper electrophoretic analysis revealed that iodide^{131} represented only a small fraction of the products of degradation by liver homogenates of insulin-I^{131} and glucagon-I^{131} (Fig. 8). Paper chromatographic analysis of butanol extracts showed that the major product of degradation migrated with

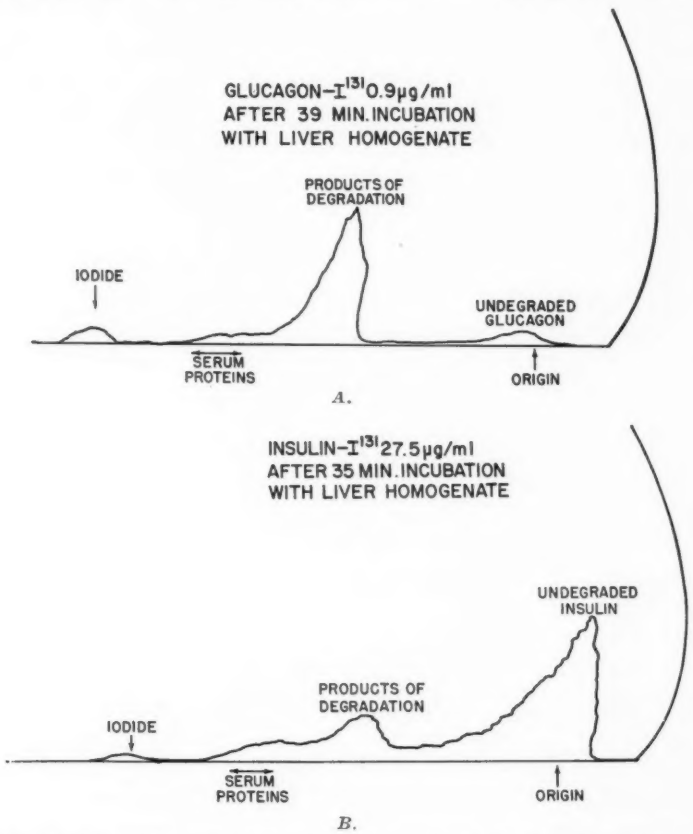


Fig. 8.—*A* and *B*, Short-run electrophoresis combined with hydrodynamic flow of glucagon-I^{131} and insulin-I^{131} after incubation with rabbit liver homogenate. Serum and Orinase were added prior to electrophoresis.

an R_f between that of monoiodotyrosine and diiodotyrosine and could not be attributed to an iodinated amino acid. It is probable that this nonprecipitable product is a small peptide. These latter studies could only be performed on samples in which further enzymatic degradation was stopped with Orinase,

*The use of this term does not necessarily imply any specificity whatsoever for this enzyme system.

since, at least in the case of the electrophoresis experiments, some further breakdown of the tagged hormones would have continued during the electrophoresis itself.

A highly lipemic serum was frequently observed among the cortisone-treated rabbits.

DISCUSSION

The rapid removal of insulin-I^{131} from the circulation reported previously for rat,[10] mouse,[11] and man[5] is likewise observed in the rabbit. However, the rapidity of disappearance of glucagon-I^{131} from the plasma, which has not been reported previously, exceeds even that of insulin. Within 10 to 15 minutes the plasma concentration of glucagon had already dropped to a level indicating an apparent space of distribution equal to that of the entire body weight and 2 to 3 times as great as that for insulin at the same time. That these low plasma concentrations reflect not simply distribution but actual degradation is indicated by the rapid appearance of I^{131}-labeled products of metabolism. To our knowledge, the appearance of an iodinated compound having the electrophoretic and chromatographic characteristics of monoiodotyrosine is the first direct evidence that I^{131} is separated from I^{131}-labeled proteins in vivo by hydrolysis rather than by simple deiodination. The analysis of the products of enzymatic breakdown in liver homogenates further validates the conclusion that removal of the I^{131} from I^{131}-labeled proteins is a manifestation of proteolytic degradation.

A more rapid metabolic degradation of glucagon than insulin is also suggested in the in vitro studies with liver homogenates which confirm similar findings with rat liver homogenates.[7] Not only is the rate of breakdown of glucagon-I^{131} greater than that of insulin-I^{131} at similar concentrations but also the maximum velocity appears to be significantly greater in the case of glucagon. Differences on a molar basis are even more marked since glucagon is the smaller molecule.

The slower disappearance of insulin-I^{131} following the administration of 500 units of unlabeled insulin is a manifestation of overloading of the insulin-degrading mechanisms which first appears only at these extremely high dosage levels. Even at the 500 unit level only a slight delay in disappearance was observed in some of the experiments, which emphasizes the tremendous capacity for degradation of increasing amounts of insulin with little change in the rate constant. From the in vitro studies it appears probable that the capacity for degrading glucagon is even greater.

With regard to the cortisone-treated rabbits, the absence of an initial hyperglycemia following the administration of "glucagon free" insulin in doses up to 100 units, reported by Volk and associates,[1] suggested that the exaggerated hyperglycemic response to commercial regular insulin was due to the glucagon contained in the insulin preparations. This was confirmed recently by evaluation of the direct response to amorphous glucagon alone[12] and it is also evident in the present studies employing crystalline glucagon (Fig. 2).

It had not been established previously whether the increased sensitivity of cortisone-treated rabbits to glucagon was related to inhibition of glucagon degradation and consequent persistence of high concentrations of circulating levels or whether the induced hyperadrenocorticism had affected the insulin-degrading mechanisms. The present studies fail to indicate any detectable alterations in the rates of metabolic degradation of insulin-I^{131} or glucagon-I^{131} in rabbits treated with large doses of cortisone. Disturbances in sugar metabolism associated with cortisone treatment in the rabbit are therefore not attributable to alterations in "insulinase" or "glucagonase" activity.

SUMMARY

1. The fate of intravenously administered insulin-I^{131} and glucagon-I^{131} was studied in cortisone-treated diabetic rabbits and in control rabbits.

2. The rate of fall in plasma protein-bound radioactivity following the administration of insulin-I^{131} was significantly slower when the labeled insulin was accompanied by doses of 500 units of unlabeled insulin rather than doses of 3 to 12 units. No differences in this respect were observed between cortisone-treated and control rabbits, although 500 units of insulin produced a prolonged initial hyperglycemia in the group treated with cortisone.

3. The rate of disappearance of plasma protein-bound radioactivity following the administration of glucagon-I^{131} at dose levels of 1.5 to 2.0 μg per kilogram of body weight was considerably faster than observed with small doses of insulin-I^{131}. There were no detectable differences in this respect between cortisone-treated and control rabbits, although a much more marked hyperglycemia occurred in the former group.

4. The rate of enzymatic degradation of insulin-I^{131} and of glucagon-I^{131}, at tracer levels and at load levels, by liver homogenates did not differ detectably for cortisone-treated and control rabbits. Liver "glucagonase" activity was significantly greater than liver "insulinase" activity in both groups of animals.

5. Electrophoretic and chromatographic analyses of plasma samples revealed that in vivo metabolic degradation of the labeled hormones is characterized by proteolytic activity, manifested by the appearance of labeled monoiodotyrosine in the plasma prior to the appearance of iodide^{131}.

6. Electrophoretic and chromatographic analyses indicated that enzymatic degradation by liver homogenates is characterized by only minimal deiodination. The major products of liver "insulinase" and liver "glucagonase" activity could not be definitely identified but they may be peptides.

7. Abnormalities in sugar metabolism which accompany the administration of large doses of cortisone to rabbits are not attributable to alterations in "insulinase" on "glucagonase" activity.

We are indebted to Mrs. Frances Whitney for technical assistance. We also wish to thank Mrs. Melanie Knopf for the illustrations, and Mrs. Frieda Steiner and Miss Eve Spelke for secretarial assistance.

REFERENCES

1. Volk, B. W., Lazarus, S. S., and Lew, H.: The Effect of Large Doses of Regular and "Glucagon-free" Insulin in the Blood Sugar of Rabbits Pretreated With Cortisone, ACTH and Growth Hormone, Metabolism **4**: 503, 1955.
2. Conn, J. W., Louis, L. H., and Johnston, M. W.: Metabolism of Uric Acid, Glutathione and Nitrogen, and Excretion of "11-Oxysteroids" and 17-Ketosteroids During Induction of Diabetes in Man With Pituitary Adrenocorticotrophic Hormone, J. LAB. & CLIN. MED. **34**: 255, 1949.
3. Conn, J. W., and Fajans, S. S.: Influence of Adrenal Cortical Steroids on Carbohydrate Metabolism in Man, Metabolism **5**: 114, 1956.
4. Bauman, A., Rothschild, M. A., Yalow, R. S., and Berson, S. A.: Distribution of I^{131} Labeled Human Serum Albumin in Congestive Heart Failure With and Without Proteinuria, J. Clin. Invest. **34**: 1359, 1955.
5. Berson, S. A., Yalow, R. S., Bauman, A., Rothschild, M. A., and Newerly, K.: Insulin-I^{131} Metabolism in Human Subjects: Demonstration of Insulin Binding Globulin in the Circulation of Insulin Treated Subjects, J. Clin. Invest. **35**: 170, 1956.
6. Yalow, R. S., and Berson, S. A.: Effect of X-rays on Trace-labeled I^{131} Insulin and Its Relevance to Biologic Studies With I^{131} Labeled Proteins, Radiology **66**: 106, 1956.
7. Yalow, R. S., and Berson, S. A.: Unpublished observations.
8. Nelson, N.: Photometric Adaptation of Somogyi Method for Determination of Glucose, J. Biol. Chem. **153**: 175, 1944.
9. Roche, J., Lissitzky, S., and Michel, R.: Chromatographic Analysis of Radioactive Compounds From the Thyroid Gland and Body Fluids. Methods of Biochemical Analysis, vol. 1, New York, 1954, Interscience Publishers, Inc., p. 243.
10. Elgee, N. J., Williams, R. H., and Lee, N.: Distribution and Degradation Studies With Insulin-I^{131}, J. Clin. Invest. **33**: 1252, 1954.
11. Mirsky, I. A., Perisutti, G., and Jinks, R.: The Destruction of Insulin by Intact Mice, Endocrinology **56**: 484, 1955.
12. Volk, B. W., Lazarus, S. S., and Lew, H.: Unpublished observations.

THE SIMULTANEOUS OCCURRENCE OF RHEUMATOID ARTHRITIS AND AGAMMAGLOBULINEMIA

ROBERT A. GOOD, M.D.,* JEROME RÖTSTEIN, M.D., AND
WILLIAM F. MAZZITELLO, M.D.
MINNEAPOLIS AND ST. PAUL, MINN.

AGAMMAGLOBULINEMIA[1] is now a well-defined clinical entity which occurs in two forms. In one form, occurring only in male children, the disease appears to be the result of an inborn error of metabolism transmitted as a sex-linked recessive trait.[2] In the other, the dysmetabolism of gamma globulin appears to be acquired beginning in either sex at any age.[3] Clinically, both diseases are featured by the repeated occurrence of bacterial infections.[1,4] Particularly troublesome in these patients have been bacterial respiratory infections, meningitis, septicemia, and bacterial infections of the urinary tract, skin, and bowel.[1, 2, 4, 5]

Extensive immunologic studies have indicated that the enhanced susceptibility to bacterial infection characteristic of these patients is due to a generalized immunologic unresponsiveness.[2, 4] Thus, it has been shown that the children with agammaglobulinemia lack natural antibodies and antibodies present in most normal persons as a consequence of immune response to antigens ubiquitous in nature. In addition, these patients fail to form circulating antibodies in response to the most intensive antigenic stimulation. In adults with acquired agammaglobulinemia there likewise exists a profound susceptibility to bacterial disease. Although serums of both the adults with acquired agammaglobulinemia and the children with the congenital form of the disease are completely lacking in gammaglobulin as revealed by both free and zone electrophoresis, analysis by a precise and delicate immunochemical method indicates that in neither instance is the agammaglobulinemia complete.[6] Instead, minute amounts of globulin immunochemically identifiable as gamma globulin are present in the childrens' serum and slightly larger amounts are present in the serum of adults with electrophoretically identifiable agammaglobulinemia.[6 7] Similarly, whereas patients with congenital agammaglobulinemia are completely unresponsive immunologically, a feeble immune response sometimes can be identified in adults with the acquired disease.[4, 8] From the standpoint of precise terminology none of the 58 patients thus far described can be said to have complete agammaglobulinemia. Although it

From the Pediatric Research Laboratories of the Variety Club Heart Hospital, the Department of Medicine, University of Minnesota, Minneapolis, and Ancker Hospital, St. Paul.

Aided by grants from the U. S. Public Health Service, the Helen Hay Whitney Foundation, the American Heart Association, the Minnesota Heart Association, and the Otto Bremer Foundation.

Received for publication July 12, 1956.

*American Legion Memorial Heart Research Professor of Pediatrics.

would seem that hypogammaglobulinemia provides a more accurate description of these cases, it is likely that the term agammaglobulinemia, originally employed by Bruton, will be retained.

Studies of the survival time of gamma globulin and antibody injected parenterally in these patients lends substantial support to the concept that the extremely low levels of antibody and gamma globulin in these patients is a function of failure of their formation and not to inordinate decay, destruction, or loss of these substances.

Investigation of agammaglobulinemia as an "experiment of nature" has already provided much insight into the relationship of gamma globulin formation to other metabolic, morphologic, and immunologic phenomena. Because of the unique opportunity afforded by the availability of patients whose only demonstrable metabolic disorder is their failure to synthesize gamma globulin and antibody at a normal rate, much additional information concerning the role of gamma globulin, circulating antibody, and the immune response in the body economy can be anticipated from study of patients with this disease. Certainly, at the present time, no comparably incisive model for the analysis of immunologic phenomena and the relation of immunologic to clinical and pathologic events is available in the experimental laboratory.

Consequently, in the course of our study of a series of 11 patients with agammaglobulinemia and our review of the reports appearing in the literature on similar cases, we have sought instances in which events observed in agammaglobulinemic patients might contribute to the understanding of mechanisms involved in expression of, or defense against, the diseases which occurred in these cases.

Arthritis appears to occur with inordinate frequency in patients with both congenital and acquired agammaglobulinemia and in many instances cannot be shown to have a basis in direct infection of the joints.[1, 9, 14] The clinical and laboratory findings of rheumatoid arthritis in 3 of our patients and 5 other patients from the literature, comprising a total of 58 reported cases, appear to provide provocative information concerning the etiological and pathogenic mechanisms responsible for rheumatoid arthritis. Indeed, the development of a syndrome indistinguishable from classical rheumatoid arthritis in patients with agammaglobulinemia who have virtually complete immunologic unresponsiveness provides an incisive critique of the concept that rheumatoid arthritis results from an immunologic response. The observations further render untenable the concept that this disease is caused by the presence of excessive amounts or qualitatively abnormal gamma globulin in the serum of patients with rheumatoid disease.

RESULTS

In Table I are summarized the published observations which reveal the frequent occurrence of joint disease in patients with congenital and acquired agammmaglobulinemia. As will be noted in the table, arthritic manifestations are a common clinical feature of patients with agammaglobulinemia. While in some of the reported cases these manifestations could not be distinguished from the manifestations of classical rheumatoid arthritis, in others a noncommittal

diagnosis of idiopathic tenosynovitis was made, and in still others the arthritic manifestations, although not due to septic involvement of the joint, appeared clinically to be associated with the occurrence of a septic process.

TABLE I

	AUTHOR AND REFERENCE	YEAR	SEX	AGE	ARTHRITIC MANIFESTATIONS
CONGENITAL AGAMMAGLOBULINEMIA	Bruton, O. C.[1]	1952	Male	8 years	Two attacks of arthritis of the left knee and left shoulder. The joints revealed only tenderness to motion. At the time of the episodes, fever and leukocytosis were present
	Bruton, O. C., Apt, L., Gitlin, D., and Janeway, C. A.[33]	1953	Male	Approx. 9 years	Mention of occurrence of arthritis and tenosynovitis in some cases
	Hayles, A. B., Stickler, G. B., McKenzie, B. F.[10]		Male	9 years	Frequent attacks of inflammatory arthritis
		1954	Male	9 years	Since the age of 18 months the patient had many episodes of febrile illness associated with tenderness, limitation of motion, and swelling of joints; he obtained relief with antibiotics
	Fischer, C. C.[9*]	1955	Male	5 years	Several episodes of pain and swelling of the right knee, left hand, and left elbow. Synovectomy of the right knee joint performed. Chronic inflammatory process
	Von Kulneff, N., Pederson, K. D., Waldenstrom, J.[11]	1955	Male	10 years	One attack of septic arthritis, 1 attack of acute arthritis with pain in the left hip. This episode was not accompanied by fever but there was an elevated ESR
ACQUIRED AGG.	Grant, F. H., and Wallace, W. D.[12]	1954	Female	20 years	Pain in the hands and wrists which resembled early rheumatoid arthritis
	Lang, N., Schettler, G., and Wildhack, R.[13]	1954	Female	29 years	Had joint disease of the left hip joint and developed a Bucken contracture
	Collins, H. D., Dudley, N. R.[14]	1955	Female	53 years	Two episodes of pain and swelling of the left knee and pain in the left hip without chills or fever. After 2 months of antibiotic therapy the patient was able to walk with crutches

*Same case as number 3 reported in this paper.

In our own material, 3 patients have had a disease indistinguishable from rheumatoid arthritis. In 2 instances the illness was diagnosed as rheumatoid arthritis by competent internists and rheumatologists who had no knowledge of the existence of the underlying serologic disorder. In both of these patients who suffered from acquired agammaglobulinemia the clinical onset of agammaglobulinemia preceded the clinical onset of arthritis. Our third patient, a 6-year-old child with congenital agammaglobulinemia, previously reported by

Fischer,[9] began to have severe joint disease at 3 years of age. The onset of arthritis and chronic synovitis in this instance began two and one-half years after the onset of the clinical expression of his agammaglobulinemia. As in all cases of agammaglobulinemia thus far reported, the agammaglobulinemic state was featured in each of these patients by many recurrences of severe life-threatening bacterial infections.

CASE REPORTS OF AGAMMAGLOBULINEMIC PATIENTS WITH RHEUMATOID ARTHRITIS

F. H.: In 1949, at the age of 54 years, this patient began suffering from recurrent attacks of pneumonia. These recurrences of pneumonia continued until the time of his death in 1954. During the period of his clinical illness he suffered from pneumonia on at least 17 different occasions. During the summer of 1951 he noted the gradual onset of swelling, pain, and stiffness in both wrists. At this time a mediastinal tumor was discovered and upon removal it was classified histologically as a thymoma. A serum protein determination done just prior to surgery was 5.1 Gm. per cent. After surgery a four-month remission of joint symptoms occurred. Thereafter a gradual recurrence of symptoms took place. On several occasions during 1952 until mid-1954 the wrists, left elbow, metacarpal-phalangeal, and mid-phalangeal joints exhibited

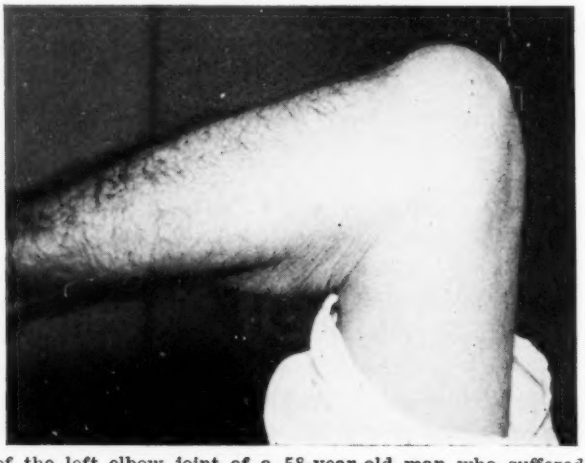
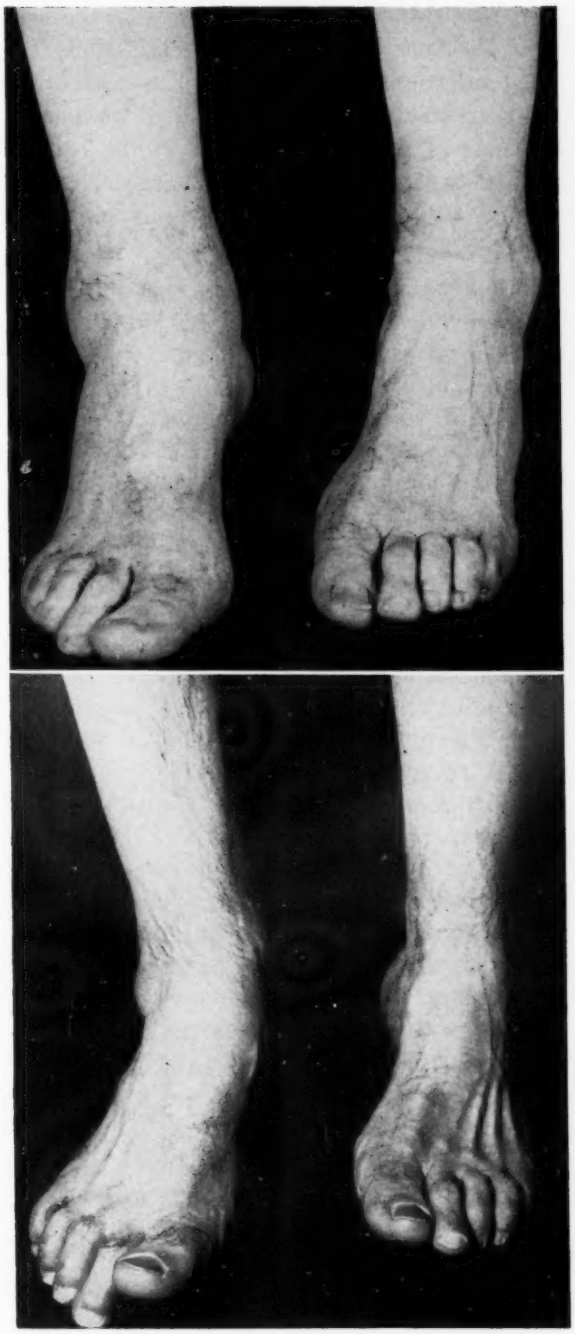


Fig. 1.—Swelling of the left elbow joint of a 58-year-old man who suffered from both agammaglobulinemia and rheumatoid arthritis.

swelling, heat, erythema, and tenderness. Usually the sedimentation rate was moderately elevated (30 to 45 mm. per hour Westergren) without a marked leukocytosis. If one of the many episodes of pneumonia occurred during a recrudescence of acute rheumatoid disease, the patient experienced relief of joint symptoms during the height of fever, the relief usually lasting for about one week. A rheumatologist at the University Hospitals* saw the patient in consultation on April 29, 1954, at which time the patient's physical findings were recorded as follows: bilateral tender wrist joints with limitation of dorsiflexion to 40 degrees, swollen and tender second left mid-phalangeal joint, and thickened capsule of the left knee with a flexion contracture. His diagnosis was rheumatoid arthritis.

*Dr. Paul Bilka, Director of the Arthritis Clinic, University of Minnesota Hospitals.

A.



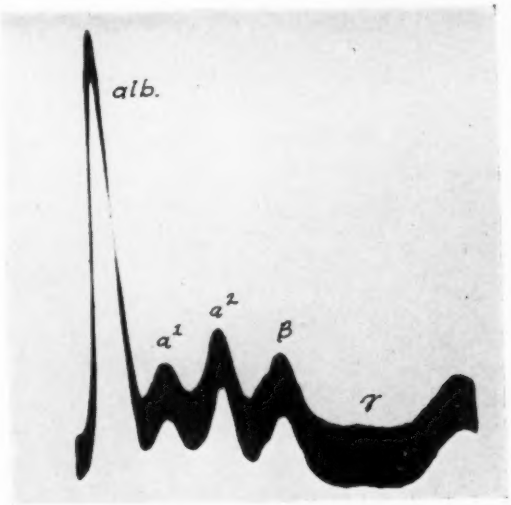
B.

Fig. 2.—Improvement in arthritic manifestations in an agammaglobulinemic patient following developement of jaundice due to hepatitis. In *A* note the marked swelling of the right ankle, which has completely disappeared 4 days after the onset of jaundice, *B*.

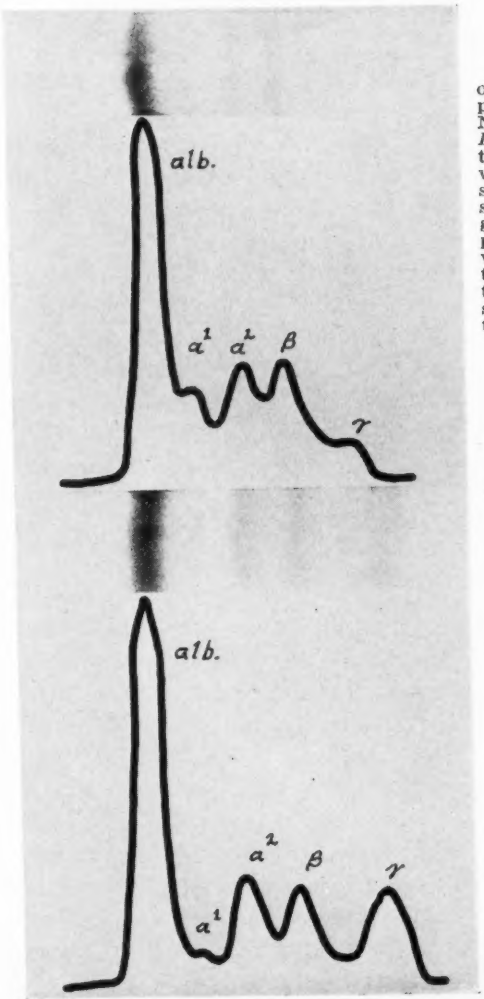
Swelling, redness, and tenderness of the left elbow occurred frequently and on several occasions rather marked effusion into the left elbow was noted (Fig. 1). Much of the time while he was being studied at the University Hospitals, swelling, tenderness, and limitation of motion of the ankles occurred which was most marked on the right. The patient at times was troubled with stiffness and tenderness of most of the joints of the body, especially ankles, knees, and hips, which was especially troublesome on arising, but gradually diminished as the day wore on. Roentgenograms of the joints revealed destructive changes and osteoporosis consistent with rheumatoid arthritis.

On December 13, 1954, the patient developed jaundice after a two-week period of anorexia and one day of nausea and emesis. Three days prior to the onset of jaundice complete relief of joint symptoms occurred. This remission of the rheumatoid arthritis continued throughout the period of the attack of viral hepatitis, which was terminal. In Fig. 2 is illustrated the dramatic improvement in the arthritis which occurred following the development of jaundice in this patient. That the agammaglobulinemia was present at the time of the onset of rheumatoid arthritis is indicated by two observations. The patient developed the recurrent episodes of pneumonia preceding the onset of his joint disease, and the value of the serum protein determination performed at the time of the first hospitalization, when his rheumatoid symptoms were just beginning, was 5.1 Gm. per cent, a value identical to that observed when the agammaglobulinemic state was known to exist. Both zone and free electrophoresis studies revealed a total absence of gamma globulin (Fig. 3). Zinc sulfate turbidity was 0 units. Immunochemical determination of gammaglobulin concentration revealed a value of 40 mg. per cent (normal 700 to 1,300 mg. per cent). Immunologic studies revealed absence of antibodies to antigens ubiquitous in nature including a positive Schick and Dick test, absence of antistreptolysin O, antistreptokinase, antihyaluronidase, and antiribonuclease, and absence of antibodies against Herpes and all three types of poliomyelitis virus. The patient was of blood group A and had the extremely low titer of isoagglutinins against group B cells of ½ (normal geometric mean titer was 1:179). Further evidence of the extreme immunologic handicap was obtained when the patient failed to respond to antigenic stimulation by antibody production against a wide variety of potent bacterial antigens.[4] The patient's bone marrow and lymph nodes showed a deficiency of plasma cells and did not respond to antigenic stimulation with plasma cell production.

L. L.: This is a 31-year-old white woman who had enjoyed good health up to nine years prior to this study. At that time, without any apparent cause, she began to experience repeated pulmonary infections including lobar pneumonia, bronchopneumonia, and bronchitis. She has had a total of 36 distinct episodes of pneumonia. During this same period, 4 episodes of pneumococcal meningitis were recorded, as were numerous instances of septicemia, sinusitis, and otitis media. Because of the recurrent infection, an electrophoretic pattern was performed and a diagnosis of agammaglobulinemia of the acquired type was established.



A.



B.

Fig. 3.—*A*, Free electrophoretic pattern on Patient F. H., an agammaglobulinemic patient who developed rheumatoid arthritis. Note absence of gamma globulin component. *B* compares the zone electrophoretic pattern of the agammaglobulinemic patient with that of an immunologically normal person with rheumatoid arthritis of comparable severity. Note the complete absence of gamma globulin in spite of the arthritic process in the agammaglobulinemic patient whereas the patient with rheumatoid arthritis shows the characteristic elevation of the gamma globulin fraction. Both patients show increase in the alpha-2 globulin fraction.

In 1947, during the time of her initial admission to the hospital for treatment of an attack of pneumonia, splenomegaly was noted in conjunction with generalized lymphadenopathy. In 1948, hepatomegaly was noted for the first time. Anemia and leukopenia were observed in 1950. Of particular interest in this case was the initial appearance of arthritis involving the right wrist and fingers of the right hand in November of 1951. Because of the combination of arthritis, anemia, leukopenia, and splenomegaly, a diagnosis of Felty's Syndrome was made by the physicians responsible for her care at that time. Splenectomy was performed which resulted in reversion of the peripheral blood picture to normal. During the following weeks while she was being studied in the out-patient department, redness, pain, and swelling of the left shoulder, elbows, and hands were noted. The observers all attributed her symptoms to rheumatoid arthritis at this time.

The most prominent manifestation of arthritis and arthralgia in this patient have occurred within the last three years. During this time she has had almost continuous involvement of the right thumb with persistent swelling, tenderness, and intermittent erythema, and limitation of motion. A similar involvement of the second interphalangeal joint of the right index finger was present during the winter of 1955. Pain in the interscapular region, left sacroiliac region, has been intermittently present for about three years. In addition, bilateral acromioclavicular bursitis developed which was continuous throughout 1953 and 1954, resulting in marked limitation of shoulder motion. During the last two years spontaneous improvement of these abnormalities has occurred.

In the fall of 1955, this patient was hospitalized for treatment of pneumonia in the right lower lobe. During this period of hospitalization, prominent swelling over the dorsum of the left hand occurred which was associated with arthralgia and limitation of motion of the wrist. Redness, swelling, pain, and erythema in the distal interphalangeal joint of the right thumb was again a problem at this time. The pneumonia responded quickly to antibiotic therapy and the patient was discharged from the hospital with persistence of the arthritis and synovitis. During the following month she experienced marked soreness, stiffness, and limitation of motion, and tenderness of both knees lasting for a period of two weeks.

At the present time the patient is troubled with arthritis of the intertarsal joints of the left foot and the metacarpal phalangeal and interphalangeal joints of the left hand.

X-ray studies of the hands and spine have not shown any osseous lesions to date. With each episode of arthritis, however, moderate elevation of the sedimentation rate has been present. Fortunately, although the arthritis has been an almost continuous problem during the past three years, no true bony deformities have as yet occurred.

B. H.*: This is a six-year-old child who was born after an uneventful pregnancy and delivery. Family history was negative. Mother, father, and 2 female siblings were normal. In the neonatal period the patient developed impetigo which responded to treatment with penicillin. However,

*This case was previously reported by C. Fischer.[9]

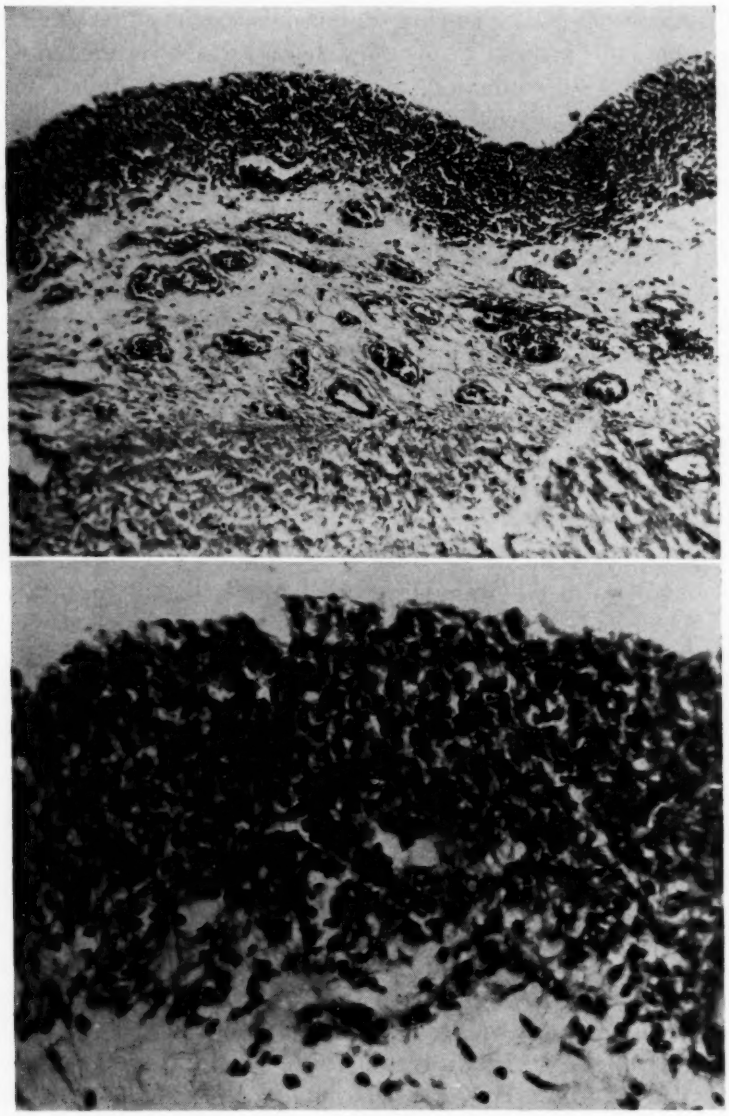
following cessation of treatment the skin disease recurred and, in spite of repeated antibiotic therapy, recurrences took place 11 times during the first year. The impetigo finally abated following intensive systemic antibiotic therapy.

During the second six months of the first year the patient had repeated episodes of gastroenteritis. During the second year he suffered many episodes of otitis media and bacterial respiratory disease which responded to treatment with penicillin and sulfonamides. During the third year he had numerous attacks of respiratory disease characterized by high fever which on each occasion responded to treatment with penicillin and sulfonamides. In June, 1951, he fell and sustained an abrasion of the left knee. He developed mild local skin infection followed by a febrile illness thought to be septicemia. He was treated with antibiotics including penicillin, terramycin, and Aureomycin, and after a stormy course, recovered. In December, 1951, when he was almost 3 years old, he developed for the first time, swelling, pain, redness, and tenderness of the left knee. The joint disease gradually subsided without specific treatment but in June 1952, he had to be hospitalized because of the development of swelling, pain, and tenderness of the right knee. A tentative diagnosis of chronic rheumatoid arthritis was made at that time. The symptoms gradually subsided again only to recur in September, 1953, with intense pain, swelling, redness, heat, and effusion of the right knee joint. During this episode he also developed pain, redness, swelling, and tenderness of all the fingers of the left hand and of both elbows. He was hospitalized and the knee joint was aspirated. This revealed a yellowish, cloudy fluid which was sterile on culture and negative for tubercle bacilli on guinea pig inoculation. During the following year the patient had mumps, repeated bacterial respiratory infection, pneumonia, several conjunctival infections, and many attacks of otitis media. All of these infections responded to treatment with antibiotics.

On several occasions during his fifth year he had swelling, pain, and tenderness of his right knee. In May, 1954, Dr. E. O. Geckeler of Philadelphia performed a synovectomy on the right knee and found the joint to contain fluid characteristic of chronic inflammatory effusion and thickened synovial membrane. The pathologic diagnosis made at the time the synovia was removed was chronic synovitis (Fig. 4). Because of the recurrent infections an electrophoretic pattern was performed and a diagnosis of agammaglobulinemia was made. Immunologic studies revealed the Schick test to be positive in spite of repeated DPT immunizations, the blood group to be O with no isoagglutinins against A or B blood cells, and failure of immune response to mumps antigen, all three polio virus antigens, and typhoid paratyphoid antigen. When studied in our laboratory, both zone and free electrophoretic studies revealed complete absence of gamma globulin. The electrophoretic pattern performed on the patient's mother revealed moderate hypergammaglobulinemia for which there was no ready explanation. None of the other members of the family showed abnormalities of the serum proteins on electrophoretic analysis.

It is our interpretation that this patient suffers from congenital agammaglobulinemia, and that he has had intercurrent rheumatoid arthritis involving numerous joints of the body. In spite of synovectomy and continued therapy with gamma globulin, exacerbation of the joint disease recurred in March, 1955. Over a period of several weeks he had effusion, pain, tenderness, and

A.



B.

Fig. 4.—*A* and *B*, Reaction of the synovium removed from the right knee of a 5-year-old child with congenital agammaglobulinemia and arthritis. Note thickening and chronic inflammatory reaction of the synovial membranes. Also note areas of fibrinoid necrosis characteristic of this inflammatory process.

limitation of motion in the righ knee. Recovery again occurred spontaneously and except for occasional stiffness of the joints in the morning the child has now been free of joint disease for approximately one year.

Occurrence of Other Fibrinoid (Collagen) Diseases in Patients With Agammaglobulinemia.—In addition to these three cases of agammaglobulinemia with rheumatoid arthritis and those indicated by the case reports in the literature referred to, Janeway, and associates[15] have described the occurrence of nondiagnostic joint disease and tenosynovitis in approximately 50 per cent of the agammaglobulinemic patients which they have studied. In addition, other forms of fibrinoid disease have been observed in these patients. In a personal communication to the authors, Bowen[16] described a female child with electrophoretic agammaglobulinemia who suffers from scleroderma as well. Dr.

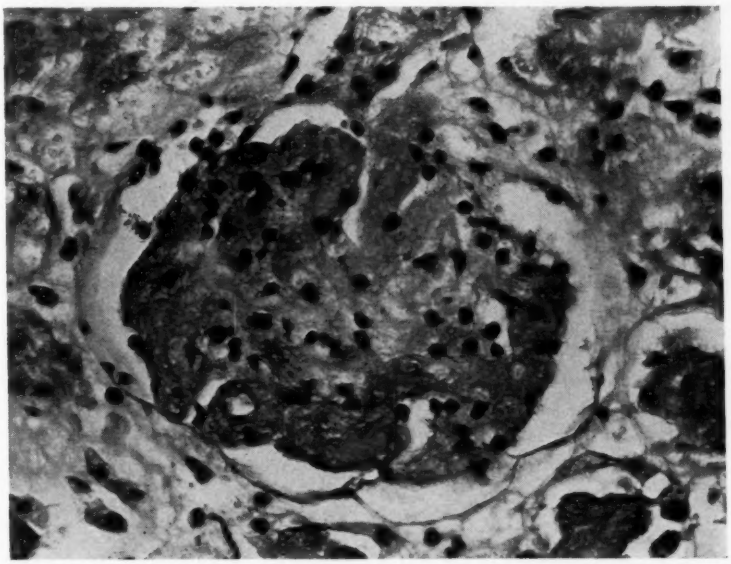
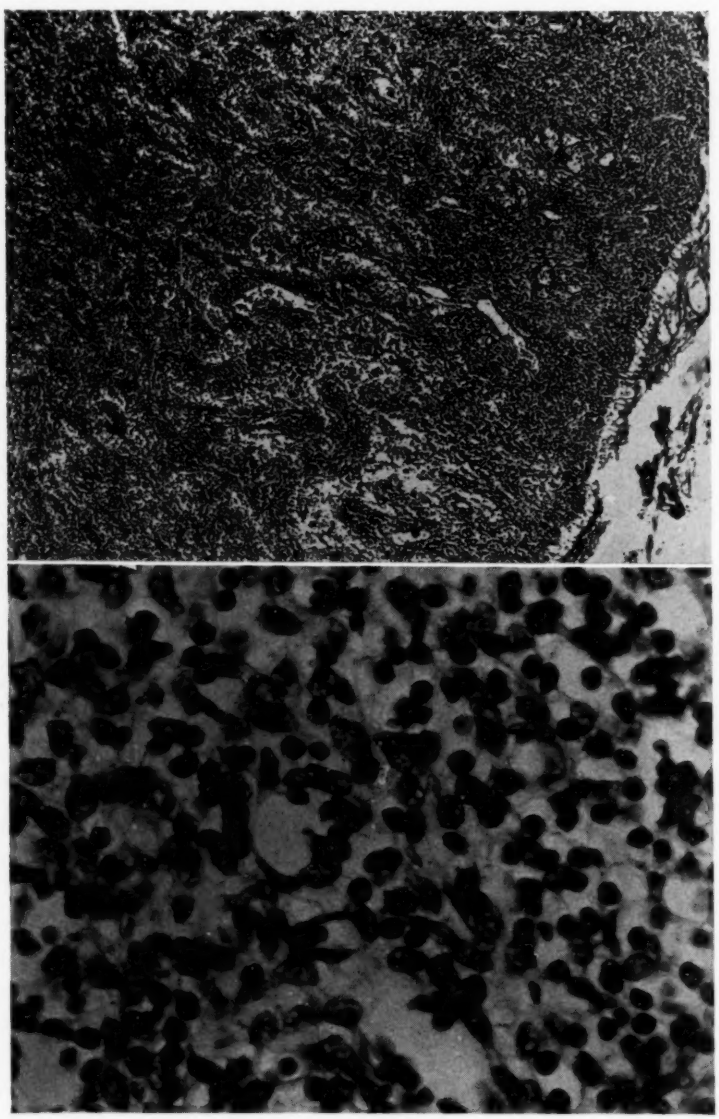


Fig. 5.—Diffuse vascular disease of the kidneys occurring in a patient with presumed aggammaglobulinemia. Note the occlusion of the glomerular capillaries with fibrinoid material.

Arild Hansen[17] and his co-workers are presently studying a patient, whom one of us (Dr. Good) has had the opportunity to observe. This patient suffers from the combination of classical dermatomyositis and complete electrophoretic agammaglobulinemia. Finally, we have recently been afforded the opportunity to study the pathologic material from a 4-year-old patient whose underlying disease might be presumed to be agammaglobulinemia. This child died of renal failure and presented pathologic findings of bilateral cortical necrosis of the kidneys and vascular changes compatible with the diagnosis of generalized collagen disease or lupus erythematosus. The patient's history is of interest. He was born after a normal gestation and delivery and was well during the first six months of life. He began to have trouble at 6 months of age with recurrent bacterial infection. He suffered from frequent respiratory infections, pneumonia, skin infections, throat infections, and otitis media. Indeed his entire life was represented by one round of severe bacterial infections after another. The terminal illness in this patient began with a skin infection following which he developed joint manifestations, a debilitating febrile disease which was not diagnosed, had renal failure, and died. In Fig. 5 is illustrated the occlusion of the glomerular capillaries with fibrinoid material and

necrosis of the renal cortex which featured the vascular disease occurring in this patient. In addition, necrotizing arteritis involving both the small- and medium-sized blood vessels was observed in this patient.

A.



B.

Fig. 6.—Structure of the lymph node in patient with presumed agammaglobulinemia who died of diffuse fibrinoid vascular disease. Note relatively thin cortex, absence of primary and secondary follicles, *A*, and absence of plasma cells on high-power view, *B*. The lymph node morphology in this patient was typical of that which characterizes agammaglobulinemia.

That this patient may have suffered from agammaglobulinemia is suggested by three observations: (1) The child had a clinical history characteristic of agammaglobulinemia, namely, the onset in the second half of the first year of a disease featured by innumerable severe bacterial infections; (2)

No plasma cells could be found on extensive cytological study of the tissues of the body obtained at post mortem; (3) The child's younger male sibling presented a clinical history identical to that preceding the terminal illness of the patient and has complete agammaglobulinemia and immunological unresponsiveness characteristic of the congenital type. In Fig. 6 are shown a low- and high-power view of the lymph node from the child who died of the generalized fibrinoid disease. The absence of follicles, the relatively thin cortex of the node, and the absence of plasma cells on the high-power study of the node are all characteristic of the lymph nodes in patients with agammaglobulinemia.

DISCUSSION

Although theories concerning the pathogenesis of rheumatoid arthritis and the several other entities often grouped together as collagen or mesenchymal diseases have been legion, no clear delineation of their nature has been forthcoming. Particularly prominent among the theories of etiology and pathogenesis have been those relating the occurrence of these diseases to hypersensitivity,[18] the formation of antibody,[19] and disturbances of gamma globulin metabolism.[20]

Dawson and Tyson[21] reviewed the early literature on the relationship of rheumatoid arthritis, rheumatic fever, and other mesenchymal diseases and concluded from the evidence available that striking similarities exist between these diseases. They believe that the differences between the two diseases were of degree rather than kind. Rich[18] enlarged on this concept and pointed out the histologic similarities between lesions of rheumatoid arthritis, rheumatic fever, periarteritis nodosa, and disseminated lupus. Using horse serum and egg albumin, Rich and Gregory[22, 23] produced serum sickness in rabbits and studied the histologic lesions resulting from this form of hypersensitivity reaction. The focal degeneration of collagen, vascular lesions, and myocarditis produced experimentally were linked to the lesions occurring in patients with the so-called collagen diseases. The protoypic lesion of this class was thought to be the nodule observed in rheumatoid arthritis. Although unable to establish the relationship positively, Rich[18] concluded from his studies that rheumatoid arthritis, rheumatic fever, and the other mesenchymal diseases have basic tissue changes similar in every way to those which occur in the reactions of anaphylactic hypersensitivity in animals and that these several diseases have in common a basis in anaphylactic hypersensitivity. Impressed by the regular elevation of serum gamma globulin concentration in rheumatoid arthritis[24] and the collagen diseases,[25] Ehrich[20] has proposed that the plasma cell proliferation and the increase in gamma globulin concentration are of etiological and pathogenic significance in rheumatic fever, rheumatoid arthritis, lupus erythematosus, and other related diseases.

The relatively recent interest in the anomalous behavior of the serum of patients with rheumatoid arthritis toward sheep red blood cells or streptococcal cells in more or less complicated systems[26-29] has provoked many investigators to consider anew etiological theories of rheumatoid arthritis which embody anaphylactic mechanisms or implicate circulating antibody at a stage in the pathogenetic process.

It is in regard to such hypotheses that we feel the observations presented in this paper are particularly pertinent. Previous studies[3-6] have established that patients with agammaglobulinemia have a gross immunologic deficit. These patients fail to produce demonstrable circulating antibodies in response to even the most intensive stimulation with a wide variety of bacterial, virus, rickettsial, cellular, and soluble antigens.[6] As a probable consequence of the immunologic failure these patients are extremely susceptible to bacterial infection. It further seems likely that the deficiency of gamma globulin characteristic of the serums of these patients is a function of their immunologic inadequacy. If, as is indicated by study of the cases reported herein, the agammaglobulinemic patient can develop rheumatoid arthritis, considerations of anaphylactic or classical immunologic mechanisms as a basis for this disease become less attractive. Indeed, we would conclude that theories invoking the production of excessive amounts of circulating antibody or gamma globulin are untenable in the face of this observation. Recent studies by Ropes and associates[29] demonstrating that the elevation in gamma globulin so characteristic of rheumatoid arthritis is, in reality, a relatively late manifestation of this disease would be in keeping with this conclusion.

Similarly the occurrence of other clinical forms of the fibrinoid (collagen) diseases in patients who have agammaglobulinemia calls into question the concept that *these diseases* have anaphylactic type hypersensitivity as their basis.

On the other hand, the recent demonstration[30-32] that patients with agammaglobulinemia possess ability to develop bacterial allergy even though they are grossly deficient in their ability to form gamma globulin and circulating antibodies would make it seem unwise to discard, without further evidence, the hypothesis that any or all of these diseases may be due to some kind of allergic reaction even though they occur together with agammaglobulinemia.

SUMMARY

1. The agammaglobulinemic patients reported in the literature who have joint manifestations possibly of a rheumatoid nature are tabulated.

2. Three patients studied in our clinic who have had agammaglobulinemia together with an illness indistinguishable from rheumatoid arthritis are presented.

3. The fact that agammaglobulinemia may occur together with other fibrinoid (collagen) diseases is mentioned.

4. The implications of these observations on theories concerning the etiology of rheumatoid arthritis and the other fibrinoid diseases is discussed.

REFERENCES

1. Bruton, O. C.: Agammaglobulinemia, Pediatrics **9**: 722, 1952.
2. Janeway, C. A., Apt, L., and Gitlin, D.: Agammaglobulinemia, Tr. A. Am. Physicians **66**: 200, 1953.
3. Good, R. A.: Agammaglobulinemia: A Provocative Experiment of Nature, Bull. Univ. of Minn. Hosp. & Minn. Med. Found. **26**: 1, 1954.
4. Good, R. A., and Varco, R. L.: A Clinical and Experimental Study of Agammaglobulinemia, Lancet **75**: 245, 1955.

5. Good, R. A., and Mazzitello, W. F.: Chest Disease in Patients With Agammaglobulinemia, Dis. Chest **29:** 9, 1956.
6. Good, R. A., and Zak, S. J.: Disturbances in Gamma Globulin Synthesis as Experiments of Nature, Pediatrics **18:** 109, 1956.
7. Gitlin, D.: Low Resistance to Infection: Relationship to Abnormalities in Gamma Globulin, New York J. Med. **31:** 359, 1955.
8. Good, R. A.: Agammaglobulinemia: An Experimental Study, Am. J. Dis. Child. **88:** 625, 1954.
9. Fischer, C. C.: Variations in Sermum Gamma Globulin, Postgraduate Med. **17:** 406, 1955.
10. Hayles, A. B., Stickler, G. B., and McKenzie, B. F.: Decrease in Serum Gamma Globulin (Agammaglobulinemia): Report of 3 Cases, Pediatrics **14:** 449, 1954.
11. Von Kulneff, N., Pederson, K. O., and Waldenstrom, J.: Drei Falle von Agammaglobulinamie. Ein klinischer, genetischer und physikalisch-chemischer Beitrag zur Kenntnis des Proteinstoffwechsels, Schweiz. med. Wchnschr. **85:** 363, 1955.
12. Grant, G. H., and Wallace, W. D.: Agammaglobulinemia, Lancet **267:** 671, 1954.
13. Lang, N., Schettler, G., and Wildhack, R.: Ueber ein Fall von Agammaglobulinemia und das Verhalten Parenteral Zugefuhiten Radioaktiv Markierten Gamma Globulins im Serum, Klin. Wchnschr. **32:** 856, 1954.
14. Collins, H. D., and Dudley, H. R.: Agammaglobulinemia and Bronchiectasis: A Report of Two Cases in Adults With Autopsy Findings, New Eng. J. Med. **252:** 255, 1955.
15. Janeway, C. A., Gitlin, D., Draig, J. M., and Grice, D. S.: Collagen Disease in Patients With Congenital Agammaglobulinemia. Sixty-ninth Annual Meeting of the Association of American Physicians, May 1, 1956. Atlantic City, N. J.
16. Bowen, R.: Personal communication.
17. Hansen, A.: Personal communication.
18. Rich, A. R.: Hypersensitivity in Disease With Especial Reference to Periarteritis Nodosa, Rheumatic Fever, Disseminated Lupus Erythematosus and Rheumatoid Arthritis, Harvey Lect. **42:** 106, 1946-1947.
19. Fischel, E. F.: The Relationship of Adrenal Cortical Activity to Immune Response, Bull. New York Acad. Med. **26:** 255, 1950.
20. Ehrich, W. E.: Immunity and Hypersensitivity Relationship to Disease in Man. Report of the Ninth M and R Pediatric Research Conference, Columbus, Ohio, M and R Laboratories, p. 54, 1954.
21. Dawson, M. H., and Tyson, T. L.: Relationship Between Rheumatic Fever and Rheumatoid Arthritis, J. LAB. & CLIN. MED. **21:** 575, 1935-1936.
22. Rich, A. R., and Gregory, J. E.: The Experimental Demonstration That Periarteritis nodosa Is a Manifestation of Hypersensitivity, Bull. Johns Hopkins Hosp. **72:** 65, 1943.
23. Rich, A. R., and Gregory, J. E.: Further Experimental Cardiac Lesions of the Rheumatic Type Produced by Anaphylactic Hypersensitivity, Bull. Johns Hopkins Hosp. **75:** 115, 1944.
24. Perlmann, G. E., and Kaufman, D.: Electrophoretic Distribution of Proteins in Serum, Plasma and Synovial Fluid of Patients With Rheumatoid Arthritis, J. Clin. Invest. **25:** 931, 1946.
25. Walker, S. A., and Benditt, E. P.: An Electrophoretic Study of the Serum Proteins in Scleroderma, Proc. Soc. Exper. Biol. & Med. **67:** 504, 1948.
26. Lamont-Havers, R. W.: Nature of Serum Factors Causing Agglutination of Sensitized Sheep Cells and Group A Hemolytic Streptococci, Proc. Soc. Exper. Biol. & Med. **88:** 35, 1955.
27. Epstein, W., and Ragan, C.: Serologic Reactions in Rheumatoid Arthritis, Am. J. Med. **20:** 487, 1956.
28. Jacobson, A. S., Kammerer, W. H., Wolf, J., Epstein, W., and Heller, S.: The Hemagglutination Test for Rheumatoid Arthritis. III. Clinical Evaluation of the Sheep Erythrocyte Agglutination and the Gamma Globulin (FII) Tests, Am. J. Med. **20:** 490, 1956.
29. Ropes, M. W., Perlmann, G. E., Kaufman, D., and Bauer, W.: The Electrophoretic Distribution of Proteins in Rheumatoid Arthritis, J. Clin. Invest. **33:** 311, 1954.
30. Zinneman, H. H., Hall, W. H., and Heller, B. I.: Acquired Agammaglobulinemia, J. A. M. A. **156:** 1390, 1954.
31. Porter, H.: Congenital Agammaglobulinemia. A Sex-Linked Genetic Trait and Demonstration of Delayed Skin Sensitivity. Abstracts of Combined Meeting of Pediatric Societies, Quebec, p. 135, 1955.
32. Good, R. A., Varco, R. L., Aust, J. B., and Zak, S. J.: Transplantation Studies in Patients With Agammaglobulinemia, Ann. N. Y. Acad. Sci. In press.
33. Bruton, O. C., Apt, L., Gitlin, D., and Janeway, C. A.: Absence of Serum Gamma Globulin, A. M. A. Am. J. Dis. Child. **84:** 632, 1953.

A SEROLOGIC INVESTIGATION OF WALDENSTRÖM'S MACROGLOBULINEMIA BY MEANS OF THE AGAR DIFFUSION METHOD

F. WESTENDORP BOERMA, PH.D.,* AND E. MANDEMA, M.D.**
GRONINGEN, THE NETHERLANDS

PRIMARY macroglobulinemia (Waldenström[1]) is a rare disease that has much in common with multiple myeloma. It is distinguished from the latter by the absence of skeletal lesions (there is at most a diffuse osteoporosis) and the more frequent appearance of moderate swelling of the lymph nodes, hepatosplenomegaly, and a hemorrhagic tendency.

In a number of recently published papers the clinical symptoms and other features of the disease are extensively dealt with.[2-13]

Electrophoretic analysis of the serum shows abnormalities comparable with those found in multiple myeloma sera.

In most cases there is hyperproteinemia, generally attributable to a great increase of γ globulin, sometimes of β globulin, or of a component with a mobility intermediate between these two. Investigations with the ultracentrifuge show the appearance of one (sometimes more than one) component with a very high sedimentation constant. The disease derives its name from these so-called macroglobulins. Sometimes such a macroglobulin will behave like a cryoglobulin, in that it flocculates at a lower temperature and dissolves again at a higher.[14] It has been a matter of consideration whether these abnormal proteins represent an increase of a protein normally present in a small quantity, or whether entirely new proteins are formed.

By means of a serologic investigation with antimacroglobulin rabbit sera Habich and Hässig[15] succeeded in demonstrating that serologically abnormal proteins are present in macroglobulinemia. An antimacroglobulin serum absorbed with normal human serum still reacts with the serum used for immunization and often too with the sera of other macroglobulinemia patients; but it no longer reacts with sera from healthy persons nor, with a single exception in which the reaction was extremely weak in addition, with sera from patients with other diseases, myeloma included. Habig and Hässig concluded from this that in these patients globulins serologically different from the normal serum proteins are present, the so-called paraproteins. Sometimes these paraproteins have an individual specificity, but for the greater part paraproteins are present which are specifically for the disease macroglobulinemia. Owing to this, cross reactions arise between the absorbed antisera and the sera of different macroglobulinemia patients. It is also possible that individually specific macroglobulins as well as macroglobulins specific for the disease are present at the same time.

Received for publication July 30, 1956.
*Bacteriological and Serological Laboratory, University of Groningen.
**Department of Internal Medicine, University of Groningen.

These investigations were confirmed by Kanzow, Scholtan, and Müting,[16] who used the same serologic method. Their antimacroglobulin rabbit serum absorbed with normal human serum reacted positively with all the sera (20) from patients with Waldenström's macroglobulinemia, whereas sera from patients with other diseases in which macroglobulins had also been formed in an abnormal quantity reacted negatively.

In order to form a clearer idea about the serologic behavior of the abnormal proteins in Waldenström's macroglobulinemia, we employed the Oudin agar diffusion method.[17-19] By this method it was found that three sera from patients with Waldenström's macroglobulinemia contained macroglobulins of different serologic specificity. Cross reactions between these sera and the absorbed antisera were not seen (Nos. 2, 3, and 4 of Table II). That the specific antibodies were indeed directed against the macroglobulin was evident because an identical result was obtained when the macroglobulin was separated out of the serum[8] and injected into a rabbit.

We have continued these investigations with another number of macroglobulinemia sera, in which, in addition to the Oudin method, the agar diffusion method of Ouchterlony[20] was applied. Rabbits were immunized with sera from seven patients with Waldenström's macroglobulinemia.* Data of the electrophoresis diagrams and the sedimentation constants are shown in Table I.

TABLE I. RESULTS OF ELECTROPHORESIS AND ULTRACENTRIFUGATION OF 7 MACROGLOBULINEMIA SERA

NAME	TOTAL PROTEIN GM. %	ELECTROPHORESIS (GM. %)									SEDIMENTATION CONSTANTS OF THE MACROGLOBULINS
		ALBUMIN	GLOBULINS								
			$\alpha 1$	$\alpha 2$	$\beta 1$	β	$\beta 2$	$\gamma 1$	γ	$\gamma 2$	
V.	9.7	3.4	0.3	0.8		0.8		1.5		2.9	15.2 S
Sp.	11.2	*									16.9 S
L.	11.1	2.9	0.4	0.8		0.9		5.5		0.6	14.9 S
B.	8.4	3.7		0.7		0.7		1.3		2.0	15.0 S
D.	7.7	2.4	0.7	1.2		1.1		1.8		0.4	14.5 S
E.	10.5	2.2	0.3	0.2	0.3		6.8		0.7		20.0 S
G.	10.8	2.2	0.35	0.7	0.5		6.7		0.35		18.4 S

*The macroglobulin in this serum precipitated at 28° C. Only paper electrophoresis could be performed. The mobility of the macromolecular cryoglobulin was that of a γ globulin.

MATERIALS AND METHODS

We immunized according to the schedule of Kabat and Mayer[21]: intravenous injections of an alum-precipitated macroglobulin serum were given during four weeks on four successive days in increasing quantities. Good results also were obtained without aluminum hydroxide. Each rabbit was injected with 1 to 1.5 ml. serum in all. The rabbits were bled a week after the last injection. The serum was filtered through a Seitz filter and preserved with 1 in 10,000 Merthiolate. Part of these sera thus obtained were absorbed with normal human serum in order to remove the antibodies against the normal serum proteins. To this end small quantities of a mixture of at least ten normal human sera, likewise sterilized by Seitz filtration and preserved with Merthiolate 1 in 10,000, were added to the antiserum until no more turbidity was observed. Before each new addition the mixture was

*We are greatly indebted to Dr. Hässig (Bern) from whom we received two of these sera; they are Nos. 3 and 16 in his publication in collaboration with Habig.

first centrifuged and often it was centrifuged after it had been kept at 4° C. during a day or a night. For the absorption of 10 ml. antiserum, quantities of normal serum were required that varied between about 2 ml. and about 4.5 ml.

For the Oudin agar diffusion, sterile tubes of 9 × 0.5 cm. were provided with a thin internal coating of agar according to the method described by Oudin. The antiserum under test was heated to about 50° C. and mixed with an equal volume of 0.6 per cent agar of the same temperature. We used Bacto powder agar dissolved in normal saline. The tubes were filled up to 2 to 2.5 cm. After the agar had solidified, the patient's sera under investigation were poured onto it. Usually these sera were diluted with an equal volume of saline. The reactions were read and, occasionally, photographed after two or three days' incubation at 22° C. or 37° C.

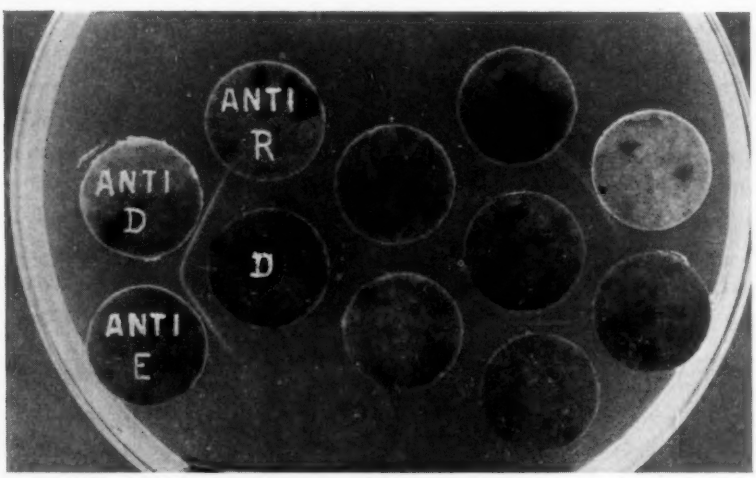


Fig. 1.—Reactions between macroglobulins and absorbed antimacroglobulin rabbit sera with filter paper discs.

In the Ouchterlony agar diffusion method a very thin agar layer is first applied to a Petri dish on which a matrix is then placed. Agar is again poured into the Petri dish. Due to the matrix, basins are formed which after solidification of the agar and removal of the matrix are filled with the antigens and antibodies to be investigated. We used about 1.5 per cent Bacto agar dissolved in normal saline. Instead of making these basins we also soaked small discs of filter paper in the solution to be investigated and placed these on a very thin agar plate. This method has the advantage that it allows working with extremely small quantities and that numerous paper discs can be placed easily on one plate. As, however, the results obtained could not be quantitatively well reproduced and as, moreover, the concentration of the antiserum was sometimes too low to produce reactions because of the diffusion from the disc into the underlying agar, we returned to the original Ouchterlony method. That good results often may be obtained with the paper discs method is shown in Fig. 1. The method is, at any rate, well suited to the obtaining of a provisional impression of the reactions between the sera and antisera. The Ouchterlony method also can be applied as follows: A 1.5 per cent agar layer 5 mm. thick is placed on a plate of photographic glass. Holes with a diameter of about 10 mm. are punched out and filled with 8 to 10 drops of the antigen and antibody solution. The antigens are diluted in such a way that the precipitation lines are as clear as possible; the point of equivalence between antigen and antibody should not be too far off. The antisera were used undiluted. After two or three days, precipitation lines will appear. The plates were usually kept in a closed

container (to prevent them from drying) for seven or ten days, after which they were photographed. This was done by placing the plate on the photographic paper in a tray filled with water.*

The results of the Oudin and Ouchterlony method are not entirely comparable. Because in the Oudin method the antigen is in direct contact with the antiserum, this method is in general somewhat more sensitive. Sometimes certain conclusions also can be drawn from the place of the zone of precipitation. We shall refer to this later. In the Ouchterlony method the solutions are much more diluted because of the diffusion than they are in the tubes, so that weak reactions may not become manifest. On the other hand, if antigen and antibody are present in about equivalent proportions the plates will keep for weeks, which may enable the manifestation of weak reactions. This method is, moreover, indispensable in the determination of identity, nonidentity, or partial identity in cross reactions.

RESULTS

Table II shows the results obtained by the Oudin method. All the absorbed antisera reacted strongly with the homologous serum. The sera anti-S and anti-V reacted exclusively with the homologous serum. The other antisera also reacted more or less clearly with some or with all of the other sera.†

TABLE II. REACTIONS IN OUDIN TUBES OF MACROGLOBULINEMIA SERA AND A NORMAL HUMAN SERUM WITH ABSORBED RABBIT ANTIMACROGLOBULINEMIA SERA

NO.	1	2	3	4	5	6	7	8
	D	Sp	L	V	B	G	E	N.S.
Anti-D	++	+	± +	±	+	±?	+	–
Anti-Sp	–	+	–	–	–	–	–	–
Anti-L	–	–	++ ±	–	±	± ± ±	± ±	–
Anti-V	–	–	–	+	–	–	–	–
Anti-B	+	±	±	±	++	±?	+	–
Anti-G	+ +	± ±	±	±	± ±	++	±	–
Anti-E	+	+	±	+	+	±?	++ ±	–

\+ One zone of precipitation.
± One weak zone of precipitation.
++ One strong zone of precipitation.
– No zone of precipitation.
When more than one zone is visible this is indicated as $\frac{++}{\pm}$ or $\frac{+}{\pm}$ etc.

A picture of an experiment is given in Fig. 2. A zone of precipitation is visible about 1 or 2 mm. below the interface of agar and the antigen solution. The reaction with normal serum was negative. These results agree with those of Habig and Hässig, and Kanzow and collaborators. Fig. 3 also shows the

*This method was adopted by us from P. Grabar, Institut Pasteur in Paris. We acknowledge our gratitude to the Z.W.O. (Netherlands Organization for Pure Research) whose generous support enabled one of us (W. B.) to visit this laboratory.

†We may remark that all our macroglobulinemia sera (12 in all) reacted positively to an absorbed antiserum, which we received from Dr. Hässig (Bern) and which also reacted positively with his macroglobulins.

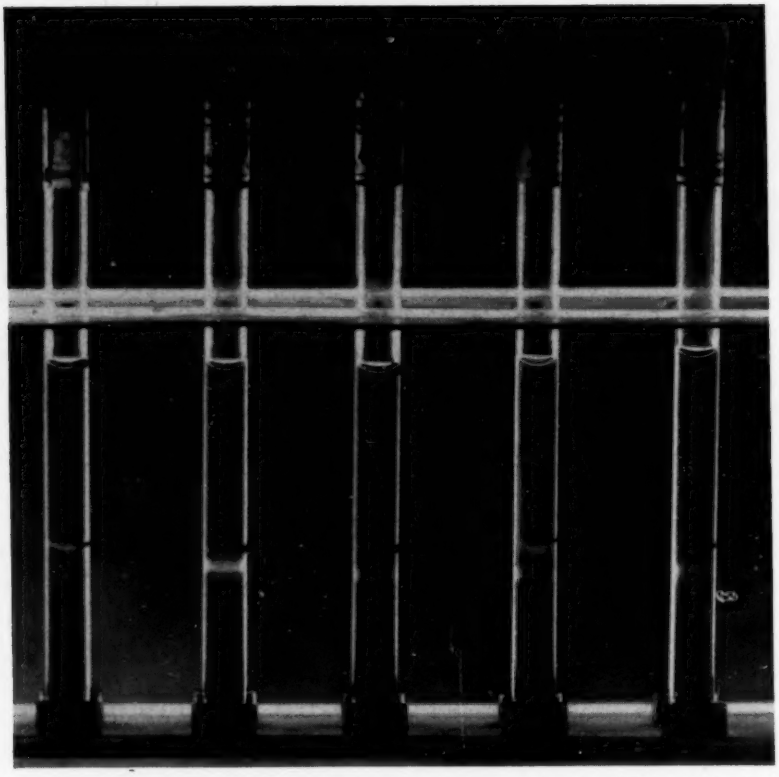


Fig. 2.—In the lower part of the tubes agar mixed with absorbed anti-B rabbit serum. In the upper part, from left to right: normal human serum and the sera of Patients B, D, E, and L.

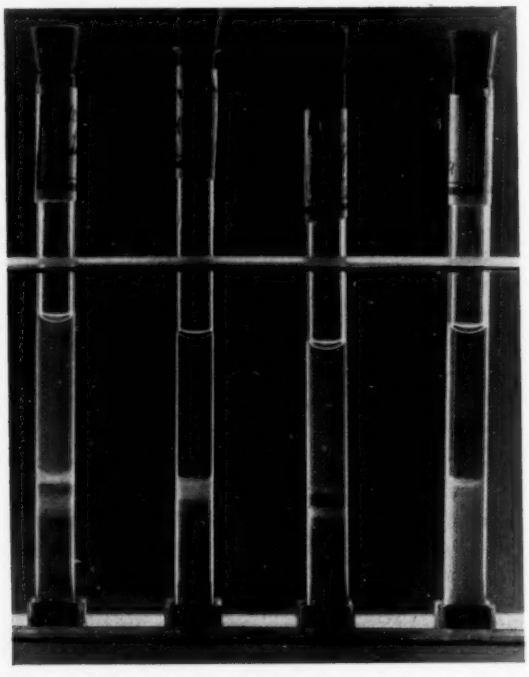


Fig. 3.—From left to right: the first two tubes contain in the lower part agar mixed with nonabsorbed anti-L serum; in the upper part, successively, serum of Patient L and normal human serum. The third and fourth tubes contain in the lower part agar mixed with absorbed anti-L serum; and in the upper part, respectively, serum of Patient L and normal human serum.

different reactions of a nonabsorbed antiserum with the homologous pathologic serum and with normal serum. The homologous pathologic serum produces a strong extra zone with a weaker one underneath, persisting after absorption. If an antiserum has not been absorbed completely, weak reactions with normal sera will arise, but these reactions always are found right under the antigen-antibody interface, whereas the zones characteristic for the macroglobulins are always found at a certain distance from this interface. Besides these characteristic zones, sometimes slight reactions also are formed right under the interface, the reaction with normal serum being *negative*. In Table II the asterisks only refer to the zones which we believe to be characteristic.

While the absorbed antisera anti-S and anti-V reacted exclusively with the homologous antigen, the sera S and V did react with the nonhomologous absorbed antisera. The same phenomenon was observed by Habig and Hässig. The absorbed antisera, anti-D, anti-B, anti-G, and anti-E, reacted more or less strongly with all the seven sera from the patients. The reaction between homologous serum and antiserum was always the strongest, which proves that all the seven sera are different.

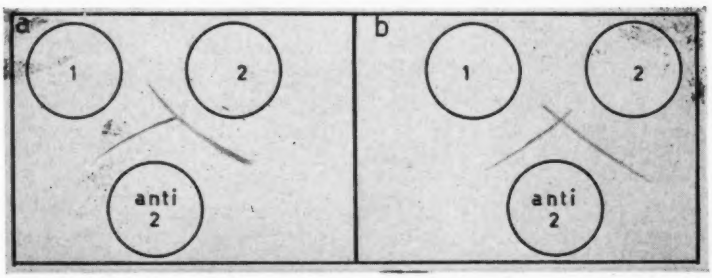


Fig. 4.—Types of reactions between an absorbed antimacroglobulin rabbit serum, the homologous serum, and a heterologous serum.

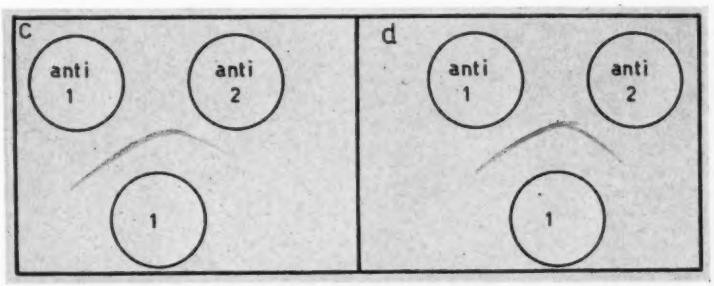


Fig. 5.—Types of reactions between an macroglobulinemia serum, the homologous antimacroglobulin rabbit serum, and a heterologous antimacroglobulin rabbit serum.

It was, however, impossible to examine with the Oudin method which relation exists between the reactions an absorbed antiserum gives with the homologous antigen and with the sera from other macroglobulinemia patients. Is there such a relationship between various macroglobulins that they are able to react with the same antibody or do there exist mixtures of paraproteins in the sera of patients some of which are related or identical in various sera and

others not? Or is it possible that various antibodies play a part here? To investigate this more closely we resorted to the Ouchterlony method. A provisional impression was obtained by soaking little discs of filter paper in antigen solutions and antisera and by placing them on a thin layer of agar, as described above. Sera and antisera which gave a distinctly visible positive reaction with this method were then examined with agar plates into which holes had been punched for the introduction of antigen and antibody solutions.

The following was observed: When an absorbed antiserum was allowed to react simultaneously with the homologous serum and a serum that had shown a cross reaction with this antiserum in the Oudin method and with the filter discs, the results pictured in Fig. 4, *a* and *b* were seen.

The course of both reactions seemed to be practically independent of each other. If, however, two different absorbed antisera were taken and if these were allowed to react with a macroglobulin serum which was homologous for one of the two and which also showed a positive cross reaction with the other, the picture changed as shown in Fig. 5, *c* and *d*.

Now there was indeed a distinct relationship between the two reactions. All the sera and absorbed antisera investigated in this manner behaved in about the same way (Fig. 6).

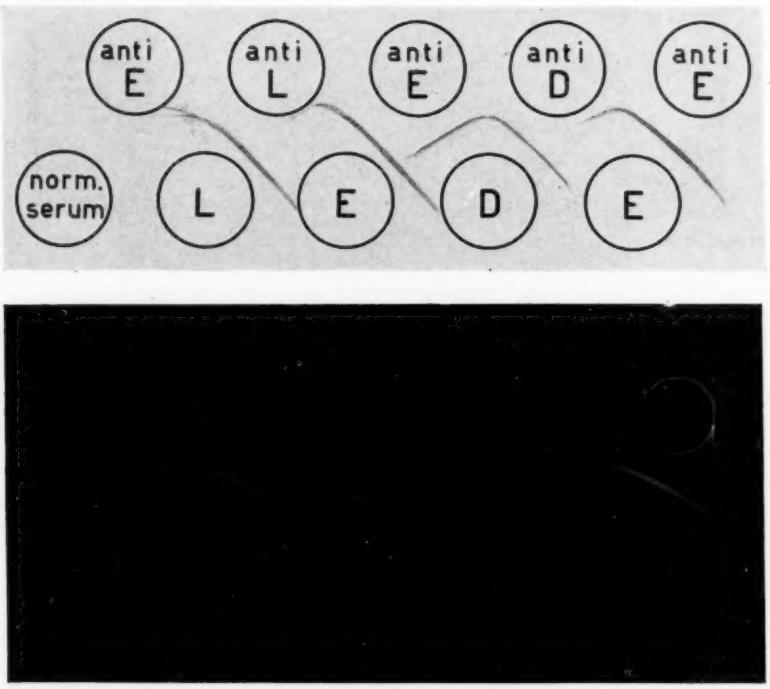


Fig. 6.—Reactions between macroglobulinemia sera and absorbed antimacroglobulin rabbit sera.

DISCUSSION

Habig and Hässig assumed that in macroglobulinemia abnormal proteins occur in the serum, which are specific for the disease. In addition to these, individually specific proteins sometimes would be present, sometimes only the

latter. However, this theory does not explain our results. If the serum from the patients contains disease-specific proteins which produce antibodies in the rabbit, then we should see cross reactions of the type of Fig. 5, *c*, both in the reaction of two different antigens with an antiserum homologous for one of the two, and in the reaction of two different antisera with an antigen homologous for one of the two. This is indeed the case if the antisera are different, but not when the antigens are different.

Our experiments with the Oudin method enabled us to conclude that all our seven macroglobulinemia sera were different. No two sera reacted exactly similarly to all the seven absorbed antisera and the reaction between homologous serum and antiserum was always the strongest. Thus we received the impression that all macroglobulins are *individually specific*, at any rate in our comparatively limited material. However, the same was stated in five sera studied after finishing this investigation. Slater, Ward, and Kunkel[22] arrived at the same conclusion with respect to the myeloma proteins. They found that in all of twenty-one myeloma patients the abnormal proteins were immunologically different.

In order to explain our results with the Ouchterlony method, we assume that when immunizing a rabbit against a certain macroglobulin, in most cases more than one kind of antibody is formed. That one antigen may cause the formation of different antibodies has been proved among others by Lapresle,[23] who found that in response to human albumin at least three different antibodies can be formed corresponding with three different antigenic groups of the albumin molecule. The same idea is found in an investigation by Ouchterlony,[24] in which different egg albumins were used and which showed that anti-egg albumin sera may contain antibodies of a different specificity. A given antibody reacts specifically with the homologous egg albumin and *not* with the egg albumin of another species, whereas another antibody may react with various egg albumins.

The same feature is observed in a recent investigation of Korngold and Lipari.[25] Antisera against multiple myeloma proteins may contain three kinds of antibodies: (1) antibodies specific for the homologous multiple myeloma protein, (2) antibodies specific for multiple myeloma proteins of the same group, (3) antibodies that can react with normal gamma-globulin and with all the multiple myeloma proteins. It is assumed that the antigen is immunologically homogeneous and that the three types of antibodies are directed against different determinant groups of the same protein molecule.

Exactly the same is found in macroglobulinemia. In the rabbit at least two different antibodies are formed against one particular macroglobulin; one of them reacts specifically with the homologous antigen, and another can react with heterologous macroglobulins, which causes cross reactions. One and the same antigen, therefore, can react with various antibodies, but conversely there are specific antibodies which react *exclusively* with the homologous antigen and others which can react to different antigens. Where Hässig assumes that the sera of patients may contain different abnormal proteins that are individually specific or specific for the disease, we are inclined to assume that there is often

but one abnormal protein and that this is individually specific. But this protein may give rise to the formation of purely specific antibodies as well as to antibodies that can react with different macroglobulins. The latter are possibly formed against certain fragments of the macroglobulin molecule, which are identical or closely related in the different macroglobulins. An antigen will react preferably with the antibody specifically directed against it.

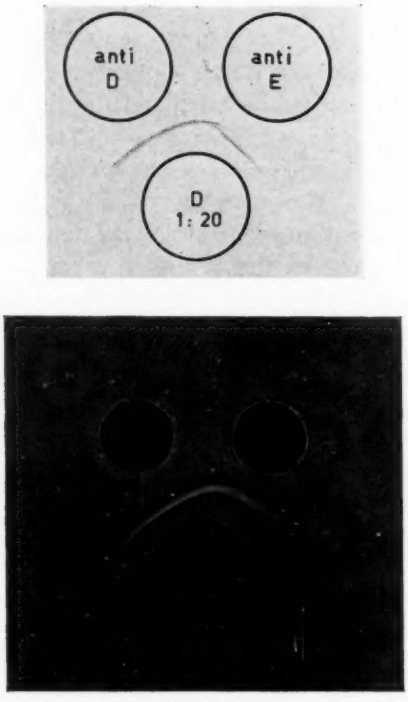


Fig. 7.—Reactions between a macroglobulinemia serum, the homologous antiserum, and a heterologous antimacroglobulin rabbit serum. Formation of a spur.

Fig. 4, *a* and *b* demonstrates that the specific antibody anti-2 reacts with 2 independently of the presence of 1. One might expect a second weaker line between anti-2 and 2, showing the reaction of the less specific antibody. This is not the case, however. Probably the concentration of this antibody is rather less; the line, therefore, should be situated to the left of the former, but no antigen is available at this site as it has already bound itself to the specific antibody. Fig. 5, *c* and *d* shows that one and the same antigen reacts with two antibodies, namely, one from anti-1 and one from anti-2, the line with the homologous antibody being the stronger. Sometimes a spur is visible (as in Figs. 5, *d* and 7). This arises from the fact that antigen 1 is not bound sufficiently by the cross-reacting antibody of anti-2; because of this, antigen 1 is still available at the right of the line 1-anti-2, which causes a slight prolongation of the precipitation line 1-anti 1 and also a slight curve, because the concentration 1 has decreased by the reaction with anti-2. In Fig. 5, *c* apparently there is not a sufficient surplus of antigen for this spur to be

Fig. 8.—Types of reactions between macroglobulinemia sera and obsorbed antimacroglobulin rabbit sera when sera or antisera are mixed. *1* = reaction E-anti-E. *2* = reaction B-anti-E. *3* = reaction B-anti-B. *4* = reaction E-anti-B.

formed. We are also strengthened in this idea by Fig. 8. In 8, *a* the antigens B and E are mixed. The line B-anti E is independent of the line E-anti E, because both antigen and antibody are different. But in Fig. 8, *b* the line B-anti E forms an arc with the line B-anti B, because now the same antigen is involved in both reactions. The same is true for the lines E-anti E and E-anti B which again form an arc. In 8, *c* the antisera anti-E and anti-B are mixed. The reactions E-anti E and B-anti B are shown independently.

This theory can also explain in a simple manner that the Sera V and S reacted to heterologous antisera, while other macroglobulins did not react to anti-V and anti-S. Apparently V and S only induced the formation of specific antibodies in the rabbit, but they themselves can react with less specific antibodies.

SUMMARY

Seven sera from patients with Waldenström's macroglobulinemia were examined serologically with the Oudin and Ouchterlony agar diffusion method. All seven sera proved to be different. In order to explain the results with the Ouchterlony method, it is assumed that in the immunization of rabbits against macroglobulins often more than one kind of antibodies are formed: i.e., one kind very specifically directed against the homologous antigen, whereas other antibodies also can react with sera from other macroglobulinemia patients.

Therefore, though all the macroglobulins possess an individual specificity, there is at the same time a certain relationship.

REFERENCES

1. Waldenström, J.: Advances in Internal Medicine, Vol. 5, Chicago, 1952, The Yearbook Publishers, Inc., p. 398.
2. Dalgaard, E. B.: A Case of Hyper- and Macroglobulinemia Accompanied by Atypical Lymphatic Hyperplasia, Acta path. et microbiol. scandinav. **27**: 506, 1950.
3. Tischendorf, W., and Hartmann, F.: Makroglobulinaemie (Waldenström) mit Gleichzeitiger Hyperplasie der Gewebsmastzellen, Acta haemat. **4**: 374, 1950.
4. Bichel, J., Bing, J., and Harboe, N.: Another Case of Hyperglobulinemia and Affection of the Central Nervous System, Acta med. scandinav. **138**: 1, 1950.
5. Schaub, F.: Zum Krankheitsbild und zur Differentialdiagnose der Makroglobulinämie Waldenström, Schweiz. med. Wchnschr. **82**: 890, 1952.
6. Kanzow, U.: Die Makroglobulinämie Waldenström, Klin. Wchnschr. **32**: 154, 1954.
7. Mandema, E.: Macroglobulinaemie van Waldenström, Nederl. tijdschr. geneesk. **98**: 2109, 1954.
8. Mandema, E., v.d.Schaaf, P. C., en Huisman, T. H. J.: Investigations on the Amino-acid Composition of a Macroglobulin and a Cryoglobulin, J. Lab. & Clin. Med. 45: 261, 1955.
9. Terwindt, W. A. M.: Macroglobulinaemie van Waldenström, Nederl. tijdschr. geneesk. **99**: 348, 1955.
10. Quattrin, N., Dini, E., e Piccoli, P.: Le porpore con ipergamma-globulinemia e la macroglobulinemia emorrhagica. Studio semiologico differenziale e patogenico, Minerva Med. **46**: 1, 1955.
11. Mackay, I. R., Eriksen, N., Motulsky, A. G., and Volwiler, W.: Cryo- and Macroglobulinemia, Am. J. Med. **20**: 564, 1956.
12. Voigt, A. E., and Flick, P. G.: Macroglobulinemia of Waldenström: A Review of the Literature and Presentation of a Case, Ann. Inst. Med. **44**: 419, 1956.
13. Jim, R. T. S., and Steinkamp, R. C.: Macroglobulinemia and Its Relationship to Other Paraproteins, J. Lab. & Clin. Med. **47**: 540, 1956.
14. Braunsteiner, H., Felkner, R., Neumayer, A., and Pakesch, F.: Makromolekuläre Kryoglobulinämie, Klin. Wchnschr. **32**: 722, 1954.
15. Habich, H., and Hässig, A.: Essai d'Analyse Antigénique des Paraprotides dans la Macroglobulinémie de Waldenström, Vox Sang. **3**: 99, 1953.

16. Kanzow, U., Scholtan, W., and Müting, A.: Serologische Differenzierung von Makroglobulinämien, Klin. Wchnschr. **33**: 1043, 1955.
17. Mandema, E., en Westendorp Boerma, F.: Serologisch onderzoek bij Multiple Myelomen (Ziekte van Kahler) Macroglobulinaemie van Waldenström en Cryoglobulinaemie, met de Agardiffusie methode volgens Oudin, Nederl. tijdschr. geneesk. **99**: 2554, 1955.
18. Oudin, J.: L'Analyse Immunochimique Qualitative; Methode par Diffusion des Antigènes au sein de l'Immunsérum Précipitant Gélosé, Ann. Inst. Pasteur **75**: 30, 1948.
19. Oudin, J.: Specific Precipitation in Gels and Its Application to Immunochemical Analysis, Methods med. Research **5**: 335, 1952.
20. Ouchterlony, Ø.: Antigen-Antibody Reactions in Gels, Acta Path. et Microbiol. Scand. **26**: 507, 1949.
21. Kabat, E. A., and Mayer, M. M.: Preparation of Antigens for Immunization and Injection Schedules, Experimental Immunochemistry, p. 543, Springfield, Ill., 1948, Charles C Thomas.
22. Slater, R. J., Ward, S. M., and Kunkel, H. G.: Immunological Relationships Among the Myeloma Proteins, J. Exper. Med. **101**: 85, 1955.
23. Lapresle, Cl.: Étude de la Sérum Albumine Humaine par un extrait de rate de Lapin. II Mise en Evidence de trois Groupements Spécifiques Différents dans le Motif Antigénique de l'Albumine Humaine et de trois Anticorps correspondents dans le Sérum de Lapin anti-Albumine Humaine, Ann. Inst. Pasteur **89**: 654, 1955.
24. Ouchterlony, Ø.: Antigen-Antibody Reactions in Gels. IV Types of Reactions in Coordinated Systems of Diffusion, Acta Path. et Microbiol. Scand. **32**: 231, 1953.
25. Korngold, L., and Lipari, R.: Multiple-Myeloom Proteins, I. Immunological Studies, Cancer **9**: 183, 1956.

SERUM PROTEIN FRACTIONS IN LYMPHOGRANULOMA VENEREUM

NINAN VERGHESE, B.Sc., M.B., B.S., M.Sc., M. P. SATYANARAYANA, B.Sc., P. N. RAMAKRISHNAN, B.Sc., B.PHARM., AND K. KUPPUSWAMY, M.Sc.
MADRAS, INDIA

LYMPHOGRANULOMA venereum (L.G.V.) is associated with hyperproteinemia and hyperglobulinemia. Gutman[1] reported hyperproteinemia in 26 out of 35 cases, of which 10 cases had total proteins ranging from 9 to 11 Gm. per cent. All cases had a reversal of the albumin globulin ratio. Other workers[2-5] have confirmed this finding. But in these cases the serum proteins have been estimated by chemical methods. With the advent of paper electrophoresis, this technique has come within the scope of ordinary clinical laboratories and the changes in serum proteins have been widely studied by this method.[6]

The available data for the amino acid composition of the serum protein fractions are very meager and any reference to the amino acid compositions of electrophoretically homogeneous protein fractions are difficult to find. The amino acid composition of pure serum protein fractions are given by Brand,[7] Shemin,[8] and Brand and Edsall.[9] The only reference we have found to electrophoretically homogeneous serum protein fractions analyzed for their amino acid composition is given by Cummings and Flynn.[10] They found no characteristic alteration in the amino acid composition of serum protein fractions in liver disease, kidney disease, or myelomatosis. Balint and Balint[11, 12] estimated tyrosine, tryptophane, cystine, arginine, and histidine in plasma proteins of patients with acute infections and found tyrosine and tryptophane decreased and cystine increased in albumin and the reverse changes in the globulins.

L. G. V. is caused by a virus infection and results in gross changes in the serum protein fractions mainly involving the gamma globulins but also the albumin and the other protein fractions. Therefore, it was considered worth while to study the serum protein fractions in L.G.V. by paper electrophoresis and to analyze the electrophoretically homogeneous albumin and gamma globulin fractions for their amino acid content.

MATERIALS AND METHODS

Samples of sera were obtained from fasting patients in the Department of Venercology of the Madras Medical College, where the diagnosis was established. In the diagnosis of L.G.V., the possible presence (concurrent or otherwise) of Kala-Azar and other diseases which are potential causes of increased globulins was excluded as far as possible. A simple electrophoresis apparatus similar to the type described by Flynn and De Meyo[13] was constructed and used for electrophoresis. Whatman No. 1 filter paper with barbitone buffer was used

From the Department of Biochemistry, Stanley Medical College, Madras.
Received for publication April 10, 1956.

for the electrophoretic runs. The samples of sera were diluted suitably with buffer solution so that the total protein concentration was reduced to about 5 Gm. per cent. About 0.015 ml. of the diluted serum was used for each electrophoretic run. This dilution helped to give a better separation of the protein fractions. One per cent bromophenol blue in ethyl alcohol saturated with mercuric chloride was used for staining the filter paper strips. Washing was done in ordinary tap water (Griffiths).[14] Five definite fractions could be delineated visually. Each fraction was cut out and the dye eluted with N/10 sodium hydroxide. The intensity of the dye was considered to be proportional to the amount of protein. The quantity of each fraction was obtained by computing the product of the per cent of the absorbed dye and the total protein content obtained by the micro-Kjeldahl method (Satoskar and associates).[15] Duplicate estimations were carried out with samples of sera with marked variations in the concentration of the serum protein fractions. The reproducibility obtained was satisfactory and compared well with the quality of reproduction obtained by Flynn and De Meyo[13] and Mackay and associates.[16]

To obtain albumin and gamma globulin fractions for the study of their amino acid composition, the above-mentioned authors used 0.4 ml. of undiluted serum on a 15 cm. broad Whatman No. 3 MM filter paper strip. Two narrow strips were cut out at either margin and stained to demarcate the protein fractions. The albumin and the gamma globulin fractions were marked out on the filter paper strip by keeping the stained filter paper strips in their original position.

Hydrolysis of the Albumin and Gamma Globulin Fractions.—The two strips carrying the albumin and the gamma globulin fractions were cut out into small bits, taken in two test tubes, and boiled with 5 ml. of concentrated hydrochloric acid, with constant shaking, until the paper bits were reduced to pulp. Then they were diluted with small amounts of water, boiled again, and filtered through Whatman No. 42 filter paper into two digestion flasks. The test tubes and the filter paper pulp were washed with small amounts of boiling water until the original concentration of hydrochloric acid became approximately diluted to 20 per cent strength. The protein solution was refluxed over a sand bath for eighteen hours using an air condenser.

Chromatography.—The hydrolysate was centrifuged and the supernatant fluid collected, along with two or three washings of the residue, and evaporated to dryness by vacuum distillation. The residue in the distilling flask was washed well with small amounts of boiling water. The washings were collected and again evaporated to dryness in a vacuum desiccator over sticks of caustic soda to remove the last traces of hydrochloric acid. The dry residue was desalted by extracting, with the required volume of a mixture of 2:3 (v/v) of butanol and phenol, so that 1 mg. of protein on the original filter paper strip would be present in 0.1 ml. of the butanol phenol mixture. This solution was centrifuged again to remove any undissolved particles. The supernatant clear fluid was used for paper partition chromatography in two dimensions; first in butanol-acetic acid and then in phenol-water. Standards for each amino acid were run in five different concentrations and in two dimensions, and the intensity of the spots was measured with a Photovolt densitometer. Graphs were prepared by plotting the products of the maximum intensity and the area of the spots against their concentrations. From these graphs the concentration of the amino acids in the chromatograms of the hydrolysates of albumin and gamma globulin were evaluated. The values obtained were expressed as grams per cent of 100 Gm. of amino acids estimated. Reproducibility that could be obtained with individual amino acids in repeated estimations varied with individual amino acids, and errors up to 15 per cent were encountered at times. Therefore, the small variations in the percentage composition of the amino acids could not be given any significance.

DISCUSSION

Table I shows the normal values for serum and plasma proteins. It will be seen that the normal gamma globulin values obtained among Indians are high when compared with those obtained in the nontropical countries. This

finding is in agreement with that of the Bombay workers (Table I). In Table II is shown the values obtained for serum protein fractions in twenty normal control subjects, 41 cases of active L.G.V. of the inguinal type, and 16 cases of healed or inactive L.G.V.

TABLE I. PLASMA PROTEIN PATTERNS IN NORMAL INDIANS AND FOREIGN GROUPS (GRAMS PER 100 ML. OF BLOOD PLASMA)

COUNTRY	NO. OF CASES	ALBUMIN	ALPHA-1	ALPHA-2	BETA	GAMMA
England (Richetts)[18]	?	4.55	0.82		0.84	0.95
U.S.A. (Keys and associates)[19]	30	4.40	0.29	0.56	0.84	0.90
India (Bombay) (Satoskar and Lewis)[20]	100	4.52	0.19	0.32	0.81	1.86*
India (Madras) (N. Verghese and associates)	20	3.96	0.39	0.61	0.88	1.49

*Gamma globulin and fibrinogen.

TABLE II. AVERAGE VALUES FOR SERUM PROTEIN FRACTIONS IN ACTIVE AND HEALED L.G.V. CASES AND IN NORMAL CONTROLS (GRAMS PER 100 ML. OF BLOOD SERUM)

TYPE OF INDIVIDUALS	NO. OF CASES	FRIE TEST	TOTAL PRO-TEIN	ALBU-MIN	ALPHA-1	ALPHA-2	BETA	GAMMA
Normal controls	20	Not done	7.26	3.96	0.39	0.61	0.88	1.49
Active L.G.V.	41	Positive	8.62	3.14	0.66	1.04	1.25	2.57
Inactive or healed L.G.V.	16	Positive	7.37	2.97	0.62	0.87	1.03	1.87

Of the 41 patients with active L.G.V., 39 had inguinal adenitis and 2 (a man and a woman) had chronic anorectal L.G.V. with stricture. Twenty-six cases out of the 41 active L.G.V. cases had total proteins above 8.32 Gm. per cent, the upper limit of normal; 40 out of 41 had total globulins above 3.91 Gm. per cent, the upper limit of normal, and 38 out of 41 had gamma globulins above 1.98 Gm. per cent, the upper limit of normal. The alpha-1 and the alpha-2 globulins were raised above the upper limit of normal in 28 cases and the beta globulins were above the upper limit of normal in 22 cases. The highest figures for serum protein fractions were obtained in the 2 cases of L.G. V. with anorectal syndrome (Table III).

TABLE III. SERUM PROTEIN FRACTIONS IN L.G.V. WITH ANORECTAL SYNDROME (GRAMS PER 100 ML. OF SERUM)

	TOTAL PROTEIN	ALBUMIN	ALPHA-1	ALPHA-2	BETA	GAMMA	TOTAL GLOBULINS
1.	11.00	3.72	0.69	0.92	1.49	4.18	7.28
2.	10.76	4.10	0.94	1.11	1.55	3.05	6.65

The 16 inactive or healed L.G.V. cases were those giving a definite history of a possible past infection of L.G.V., with the disease having healed leaving scars in the groin and a positive Frie's test. These were cases where the disease had apparently run its course without any specific treatment and the patients were asymptomatic. These cases did not show hyperproteinemia (Table

II). The average value of total proteins was only 7.37 Gm. per cent. The electrophoretic pattern showed that the globulin fractions were still above normal. The average value of total globulins was 4.36 Gm. per cent. This study confirms the findings of Rosen and associates,[17] that inactive or healed L.G.V. patients maintain a persistent hyperglobulinemia for some years.

Fig. 1 shows the normal electrophoretic pattern and the pattern obtained from one of the cases of anorectal syndrome. The amino acid composition of

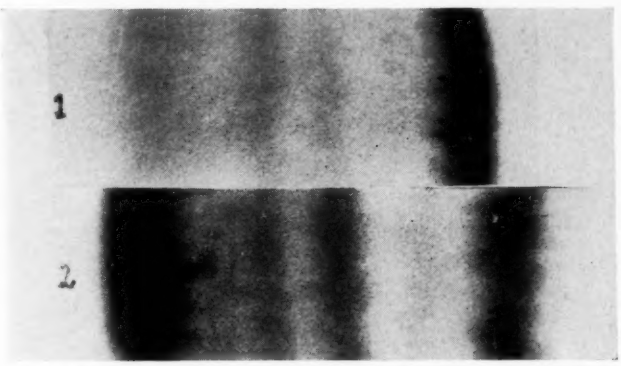


Fig. 1.—*1*, Normal electrophoretic pattern. *2*. Electrophoretic pattern from a case of lymphogranuloma with anorectal syndrome.

the albumin and gamma globulin in the two cases of active L.G.V. with anorectal syndrome was studied. Fig. 2 shows the two-dimensional chromatograms obtained with the hydrolysates of normal albumin and gamma globulin of one of the control subjects. Fig. 3 shows the two-dimensional chromatograms obtained with the hydrolysates of albumin and gamma globulin from one of the cases of active L.G.V. with anorectal syndrome. It will be apparent from the chromatograms that with the reversal of the albumin/globulin ratio in L.G.V. there appears to be a similar reversal of the amino acid composition of the albumins and the gamma globulins also. Table IV gives the percentage

TABLE IV. THE AMINO ACID COMPOSITION OF ALBUMIN AND GAMMA GLOBULIN IN NORMAL CONTROLS AND IN L.G.V. CASES WITH ANORECTAL SYNDROME (GRAMS PER 100 GM. OF AMINO ACID ESTIMATED)

AMINO ACIDS	ALBUMIN		GAMMA GLOBULIN	
	NORMAL	L.G.V.	NORMAL	L.G.V.
Leucine and isoleucine	8.9	31.3	16.8	10.4
Valine	7.3	20.8	19.7	7.7
Alanine	10.4	14.5	11.7	8.1
Tyrosine	6.7	12.0	10.2	9.4
Threonine	2.9	*	*	3.5
Glycine	4.4	1.1	1.9	7.9
Serine	6.9	0.9	2.5	8.9
Aspartic acid	2.5	*	*	2.7
Histidine	3.1	*	*	1.2
Lysine	14.2	0.9	0.7	10.0
Arginine	7.3	0.6	*	4.7
Glutamic acid	14.6	10.0	11.1	11.1
Proline	10.1	8.0	24.8	14.2

*Less than 0.5 Gm. per cent.

composition of fourteen amino acids estimated by paper chromatography and densitometry. It is seen that the amino acids which are increased in the gamma globulin are decreased in the albumin, and vice versa.

In L.G.V. there is no appreciable damage to the liver and therefore it can be expected that the synthesis of the albumins is not affected as in the liver

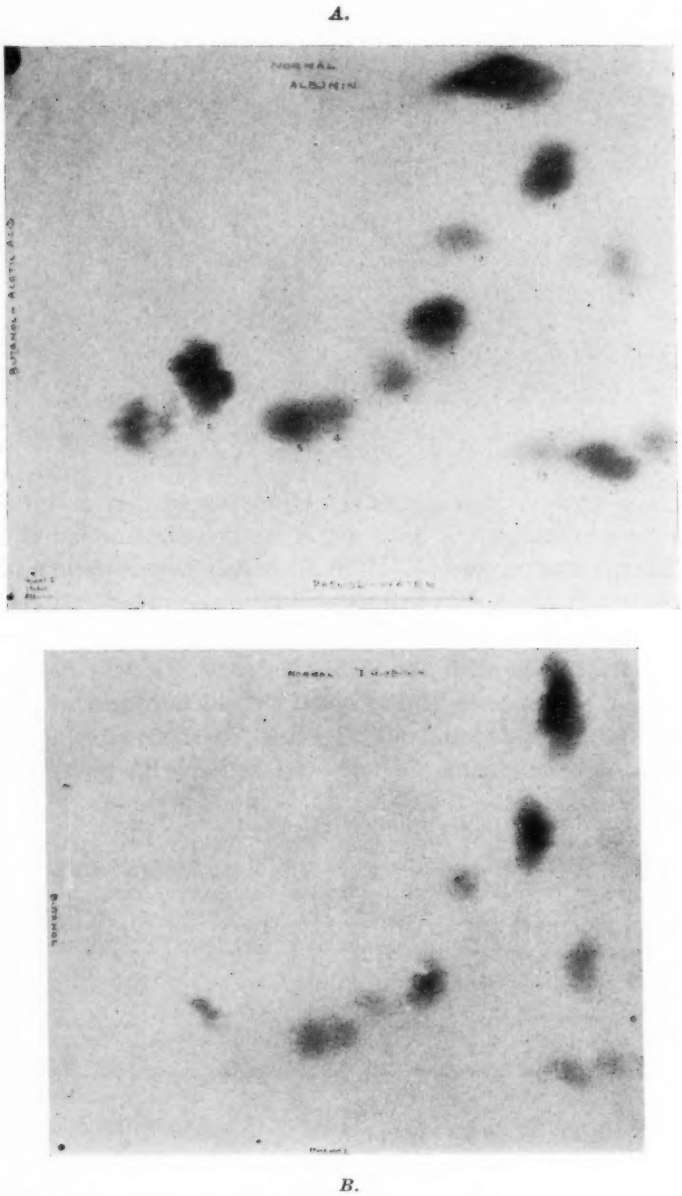


Fig. 2.—Normal control. *A*, Albumin. *B*, Gamma globulin. *1*, Aspartic acid. *2*, Glutamic acid. *3*, Serine. *4*, Glycine. *5*, Threonine. *6*, Alanine. *7*, Lysine. *8*, Arginine. *9*, Proline. *10*, Tyrosine. *11*, Valine. *12*, Leucine and isoleucine. *13*, Histidine.

diseases. It is believed that the elaboration of the antibodies in infectious diseases is a function of the gamma globulin moiety. The hyperglobulinemia in L.G.V. is probably due to the production of antibodies against the virus. It is likely that the amino acids ordinarily utilized in the synthesis of normal albumins are diverted for the production of gamma globulins in L.G.V. Incidentally, this may produce a decrease in the production of the albumin when

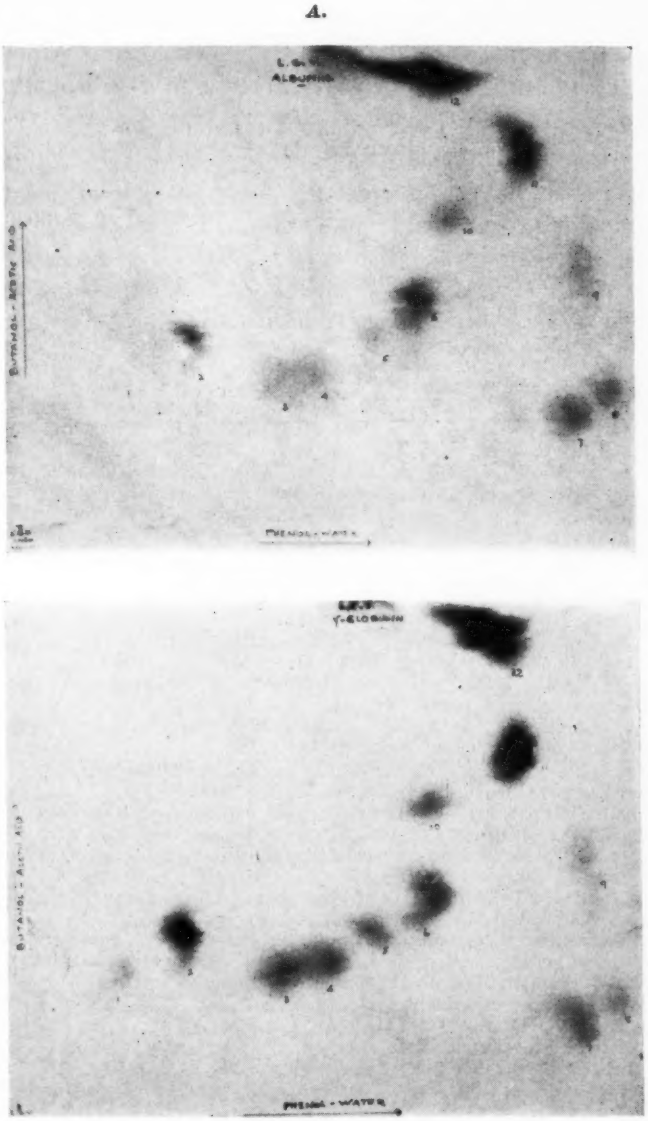


Fig. 3.—L.G.V. with anorectal syndrome. *A*, Albumin. *B*, Gamma globulin. *1*, Aspartic acid. *2*, Glutamic acid. *3*, Serine. *4*, Glycine. *5*, Threonine. *6*, Alanine. *7*, Lysine. *8*, Arginine. *9*, Proline. *10*, Tyrosine. *11*, Valine. *12*, Leucine and isoleucine. *13*, Histidine.

there is an increase in the gamma globulins. It is seen from the chromatograms that the increase in the amino acids in gamma globulin fraction is at the expense of the amino acids in the albumin fraction.

SUMMARY

The values for serum protein fractions obtained by paper electrophoresis in active and healed cases of lymphogranuloma venereum are compared with normal values in Southern Indians. The amino acid composition of electrophoretically homogeneous albumin and gamma globulin in active lymphogranuloma cases and in normal controls are presented also. In Lymphogranuloma venereum the amino acids which decrease in the albumin fraction are found to be increased in the gamma globulin fraction, and vice versa. The significance of the findings is discussed.

Our grateful thanks are due Professor R. V. Rajam, Director of the Institute of Venereology, Madras, and Dr. C. W. Chacko, Associate Professor of Serology, for their help and cooperation.

REFERENCES

1. Gutman, A. B., Gutman, E. B., Jillson, R., and Williams, R. D.: Acid Base Equivalence of Blood in Diseases Associated With Hyperglobulinemia; With Special Reference to Lymphogranuloma Inguinale and Multiple Myeloma, J. Clin. Invest. **15**: 475, 1936.
2. Gutman, A. B.: Systemic Manifestations of Lymphogranuloma Venereum With Illustrative Case Reports, New York J. Med. **39**: 1420, 1939.
3. Howard, E., Eisenman, A. J., and Straus, M. J.: Alterations in Serum Proteins in Lymphopathia Venerea, Am. J. Syph. **23**: 83, 1939.
4. Kampmeier, R. H., Smith, D. W., and Larsen, R. M.: Blood Studies in Lymphogranuloma Venereum With Special Reference to Serum Proteins, Am. J. M. Sc. **198**: 516, 1939.
5. Ira Leo Schamberg: The Course of Plasma Protein Changes in Early Lymphopathia Venereum Under Treatment With Sulphanilamide, Am. J. M. Sc. **201**: 67, 1941.
6. Franglen, G. T., and Martin, N. H.: The Use and Limitations of Filter Paper Electrophoresis, J. Clin. Path. **7**: 87, 1954.
7. Brand, E.: Amino Acid Composition of Simple Proteins, Ann. New York Acad. Sc. **47**: 187, 1946.
8. Shemin, D.: Amino Acid Determination on Crystalline Bovine and Human Serum Albumin by Isotope Dilution Method, J. Biol. Chem. **159**: 439, 1945.
9. Brand, E., and Edsall, John T.: The Chemistry of Proteins and Amino Acids, Ann. Rev. Biochem. **16**: 223, 1947.
10. Cummings, A. J., and Flynn, F. V.: Amino Acid Composition of Serum Proteins in Health and Disease, J. Clin. Path. **8**: 155, 1955.
11. Balint, P., and Balint, M.: Über die Chemische Zusammensetzung der Menschlichen Bluteiweiskorper, Biochem. Ztschr. **313**: 192, 1942.
12. Balint, P., and Balint, M.: Uber die Chemische Zusammensetzung der Menschlichen Bluteiweiskorper, Biochem. Ztschr. **315**: 41, 1943.
13. Flynn, F. W., and De Meyo, P.: Microelectrophoresis of Protein on Filter Paper, Lancet **2**: 235, 1951.
14. Griffiths, L. L.: The Electrophoresis of Serum and Other Body Fluids in Filter Paper, J. Lab. & Clin. Med. **41**: 188, 1953.
15. Satoskar, R. A., Lewis, R. S., and Gaitonde, B. B.: Electrophoretic Studies of Plasma Proteins in Virus Hepatitis, J. Lab. & Clin. Med. **44**: 349, 1954.
16. Mackay, Ian R., Volviler, Wade, Patrick D., and Goldsworthy: Paper Electrophoresis of Serum Proteins; Photometric Quantitation and Comparison With Free Electrophoresis, J. Clin. Invest. **35**: 855, 1954.
17. Rosen, I., Rosenfield, H., Bloom, D., Kransnow, F.: Lymphogranuloma Venereum: Serum Lipids and Proteins: Arch. Dermat. & Syph. **39**: 211, 1939.
18. Richetts, W. E., and Sterling, K.: Electrophoretic Studies of Serum Proteins in Virus Hepatitis, J. Clin. Invest. **28**: 1477, 1949.
19. Keys, A., Brozek, J., Henschel, A., Mickelson, O., and Taylor, H. L.: Biology of Human Starvation, Minneapolis, 1950.
20. Satoskar, R. S., and Lewis, R. A. (Unpublished)

PROTEOLYTIC ACTIVITY OF WHOLE HUMAN SERUM FOLLOWING SURGERY

WAYNE M. MEYERS, PH.D., KENNETH L. BURDON, PH.D., AND MAXINE N. RILEY, B.A.
HOUSTON, TEX.

THE role of tissue and plasma proteolytic enzymes in physiologic and pathologic processes is becoming of increasing interest, especially as it may suggest therapeutic procedures.[1] Variations during disease in the plasma protease, plasmin, or its inhibitors have been investigated rather extensively. The following scheme for the formation of plasmin in plasma by the streptococcal product, streptokinase (SK), has been elucidated by Müllertz and Lassen[2] and confirmed by Troll and Sherry[3]:

$$\text{Plasminogen proactivator} + \text{SK} \rightleftharpoons \text{Plasminogen activator}$$

$$\text{Plasminogen} \xrightarrow{\text{activator}} \text{plasmin}$$

Plasmin occurs in the euglobulin fraction of the plasma proteins; consequently this fraction is commonly used in assaying the proteolytic activity of plasma. Cliffton[4] and Scott and associates,[5] among others, using the euglobulin fraction, found that potential plasmin activity may rise following surgery. No reports on plasmin inhibitor levels in patients undergoing surgery have

TABLE I. DESCRIPTION OF PATIENTS

PATIENT	AGE	SEX	SURGERY	DURATION OF SURGERY (HOURS)
I. L.	58	F	Cholecystectomy, appendectomy, gastrotomy	1½
M. G.	71	M	Gastrectomy	3¾
F. H.	47	M	Bilateral inguinal hernia repair	1½
R. H.	44	F	Cholecystectomy, excision of breast mass	5¼
V. B.	45	M	Gastrectomy, splenectomy, appendectomy	5
O. G.	89	M	Biopsy abdominal wall, total body x-irradiation, fourth day postoperative	½
S. R.	53	F	Cholecystectomy, choleduodostomy, anastomosis hepatic artery common duct exploration	5
G. W.	19	F	Benign breast tumor excision	1
P. H.	60	F	Cholecystectomy	3
T. F.	44	F	Cholecystectomy, appendectomy	2¼
D. G.	26	F	Hemorrhoidectomy	1½
J. J.	33	M	Eye evisceration	½
A. D.	42	M	Resection and anastomosis of colon	3
R. W.	40	M	Total body x-irradiation	None

From the Department of Microbiology, Baylor University College of Medicine, Houston, Tex.

This investigation was supported in part by a grant (DA-49-007-MD-576) from the Medical Research and Development Board, Department of the Army.
Received for publication July 6, 1956.

TABLE II. SPONTANEOUS AND SK-ACTIVATED

PATIENT		PREOPERATIVE DAYS			POSTOPERATIVE HOURS				POSTOPERATIVE DAYS							
		3	2	1	3	6	9	12	1	2	3	4	5	6	7	8
I. L.	SP†	0.22	0.24	0.24	0.22	–	0.26	0.16	0.20	0.24	0.22	0.18	0.18	0.22	0.34	0.22
	SKP‡	2.12	2.22	2.20	2.32	–	2.18	2.04	1.86	1.30	1.92	1.76	2.06	1.94	2.02	2.00
M. G.	SP	0.04	0.02	0.14	–	–	0.04	0.06	0.14	0.12	0.14	0.18	0.12	0.12	0.18	0.24
	SKP	1.76	1.58	1.68	–	–	1.74	1.56	1.34	1.50	1.58	1.66	1.70	1.68	1.74	1.52
F. H.	SP	0.30	0.26	0.28	–	0.20	0.10	–	0.12	0.22	0.10	0.24	0.32	0.04	0.04	
	SKP	1.56	1.58	1.56	–	1.48	1.56	–	1.54	1.34	1.38	1.48	1.68	1.36	1.52	
R. H.	SP	0.12	0.08	0.18	–	–	–	–	0.06	0.12	0.22	0.24	0.08	0.10	0.28	0.14
	SKP	1.46	1.92	1.24	–	–	–	–	1.14	0.88	1.12	1.12	1.42	1.48	1.56	1.06
V. B.	SP	–	0.28	0.28	–	0.38	–	–	0.44	0.56	0.44	0.16	0.34	0.46	0.40	0.42
	SKP	–	1.64	1.58	–	1.70	–	–	1.58	1.32	1.22	1.16	1.30	1.40	1.42	1.52
O. G.	SP	–	–	0.22	0.14	0.30	–	–	0.30	0.32	0.24	0.18§	0.16	0.58	0.32	0.14
	SKP	–	–	1.52	1.46	1.62	–	–	1.40	1.50	1.60	1.36	1.36	1.90	1.50	1.06
S. R.	SP	0.10	0.12	0.08	–	–	0.08	0.10	0.18	0.18	0.20	0.24	0.22	0.26	0.16	0.18
	SKP	2.12	1.84	2.06	–	–	2.14	2.04	1.94	1.78	1.86	1.74	1.70	1.67	1.75	1.82
G. W.	SP	0.26	0.26	0.30	0.38	0.30	0.24	–	0.24	0.24						
	SKP	1.74	1.82	1.88	2.08	1.94	1.88	–	1.90	1.84						
P. H.	SP	–	0.20	0.12	0.12	0.22	–	0.10	0.10	0.12	0.12	0.12	0.04	0.14	0.08	0.08
	SKP	–	1.54	1.52	1.60	1.66	–	1.46	1.56	1.38	1.48	1.56	1.52	1.70	1.94	1.72
T. F.	SP	–	–	0.00	–	0.00	0.00	0.00	0.00	0.12	0.14	0.28	0.16	0.20	0.14	0.24
	SKP	–	–	2.06	–	2.12	2.02	1.90	1.82	1.78	1.88	2.02	1.96	2.08	2.04	2.12
D. G.	SP	–	–	0.18	0.16	0.06	0.14	–	0.16	0.08	0.10					
	SKP	–	–	2.22	2.12	2.24	2.18	–	1.86	2.16	2.16					
J. J.	SP	–	–	0.24	0.22	–	–	–	0.20	0.20	0.18	0.12	0.32	0.18	–	0.16
	SKP	–	–	2.26	2.24	–	–	–	2.14	2.32	2.22	2.24	2.18	2.16	–	2.22
A. D.	SP	–	–	–	0.14	–	–	–	0.22	0.44	–	0.04				
	SKP	–	–	–	1.82	–	–	–	1.88	1.86	–	1.80				
R. W.	SP	–	–	0.20	0.30	–	–	0.14	0.10	–	–					
	SKP	–	–	1.46	1.78	–	–	1.76	1.86	–	0.66					

*Expressed in units as defined in the text.
†Spontaneous protease.
‡SK-activated protease.
§Total body x-irradiation on fourth postoperative day (Patient O. G.).

been found in the literature. However, it has been demonstrated that serum trypsin inhibitors usually drop early in the postoperative period, followed by a later rise.[4] Serum plasmin inhibitors are known to increase in such conditions as rheumatoid arthritis, coronary thrombosis, pneumonia, and intestinal obstruction.[6, 7] Since Shulman[8] has pointed out that plasmin and trypsin inhibitors are distinct entities, correlations cannot be made between plasmin activity and trypsin inhibitor levels. According to Shulman, only about ten per cent of trypsin inhibition by serum is due to plasmin inhibitor, while trypsin inhibitors have no effect on plasmin.

PROTEOLYTIC ACTIVITY* OF WHOLE SERUM

POSTOPERATIVE DAYS																
9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
0.14	0.14	0.06														
1.56	1.88	1.86														
0.08	0.06	0.12	0.22	0.28	0.10											
1.58	1.24	1.04	1.60	1.64	1.76											
0.58	0.52	0.58	0.28	0.64	0.34											
1.46	1.42	1.62	1.70	1.72	1.74											
0.04	0.10	0.12	0.06	0.18	0.04	0.04	0.06	–	0.06	0.04	0.06	0.06	0.32	0.32	–	0.18
1.46	1.42	1.60	0.98	1.46	0.84	1.30	1.08	–	1.42	1.62	1.68	1.74	1.74	1.80	–	1.56
0.16	0.20	0.10	0.10	–	0.14	0.10	0.16	0.14	0.12	0.18	–	0.10	0.16	0.18	0.08	0.08
1.78	1.90	1.96	1.94	–	1.66	1.98	2.02	1.98	1.90	1.86	–	1.90	1.82	1.90	1.70	1.46
0.08																
1.72																
0.20																
2.14																

In view of the various difficulties encountered in the accurate measurement of changes in plasmin activity and plasmin inhibitor levels when the serum is fractionated, it was decided to determine plasmin activity in *whole* serum. This method has a further advantage since the proteolytic activity of serum is an expression of a series of reactions occurring under the limitations of inhibitors. Whole serum activity thus may reflect the potential situation existing in vivo more accurately than titrations of euglobulin or otherwise altered serum preparations. This present study using whole serum from surgical patients preceded the investigative work previously reported[9] in which the characteristics of the SK-activated proteolytic activity of whole serum were outlined. Consequently, certain aspects of this study did not represent optimal conditions; however, all of the specimens were assayed

TABLE III. ANTITRYPSIN VALUES

	PREOPERATIVE			POSTOPERATIVE											
	DAYS			HOURS				DAYS							
	3	2	1	3	6	9	12	1	2	3	4	5	6	7	8
I. L.	12.18*	11.88	11.22	10.71		10.71	10.59	10.71	10.71	11.37	11.13	10.23	10.98	10.59	11.37
	100†	97.6	92.5	88.2		88.2	87.0	88.2	88.2	93.4	91.9	84.5	90.2	87.0	93.5
M. G.		8.4	8.4			8.3		8.1	8.1	8.2	8.2			8.3	7.9
		100	100			98.8		96.5	96.5	97.5	97.5			98.8	94.1
V. B.		12.00	13.50		12.30			12.30	12.90	12.30	12.45	12.72	12.15	13.26	12.45
		88.9	100		91.2			91.2	95.5	91.2	92.2	94.2	90.0	98.2	92.2
O. G.			9.71		9.96			10.17	9.96	10.17	9.42	9.72	9.60	9.27	
			95.5		98.4			100	98.4	100	92.6	95.6	94.5	91.2	
S. R.	14.73	14.85	14.46			13.95	13.80	14.34	14.34	14.34	13.71	13.80	13.95	14.34	14.22
	99.5	100	97.2			94.0	93.0	96.8	96.8	96.8	92.6	93.0	94.0	96.8	95.7
T. F.			9.54		8.79	9.09	8.25	10.20	9.90	9.27	10.44	10.71	9.66	10.71	11.07
			86.1		79.4	82.0	74.5	92.2	89.2	83.6	94.3	96.9	87.1	96.9	100
J. J.			11.10	11.64				11.82	11.46	11.46	12.12	11.46	10.83		11.10
			91.5	96.0				97.5	94.4	94.4	100	94.4	89.2		91.5

*μg trypsin inhibited.

†Per cent of maximum trypsin inhibitor values over the period covered by this table.

under standardized procedures. Serum protein concentration, spontaneous proteolytic activity, and trypsin inhibitor levels also have been assayed to determine possible correlations.

METHODS

Specimens were collected aseptically, allowed to clot, and the serum separated within 12 to 18 hours after bleeding.* All sera were maintained in a frozen state until used, with a maximum storage period of 3 months. The determinations in a given assay procedure on each patient all were made at the same time, and in duplicate.

Proteolytic Activity.—Kunitz' tryptic assay procedures[10] were adapted to the measurement of proteolytic activity using casein† as a substrate. Each digestion mixture contained the following components: one ml. of 1:2 diluted serum, one ml. 6 per cent casein, 0.2 ml. SK‡ (2,000 units), and 0.8 ml. buffer. All solutions used in the digestion were prepared in M/10 phosphate buffer (pH 7.4) and carefully adjusted to pH 7.4 before use. The casein solution was boiled for 10 minutes to assure destruction of possible proteases in the preparation. A 90-minute incubation period at 37° C. was used. Activity was stopped at the end of the incubation period by adding 3 ml. of 5 per cent trichloroacetic acid (TCA) to each tube. After standing 30 minutes, the TCA mixtures were filtered through Whatman No. 42 paper, and the degree of proteolysis determined by measuring the acid-soluble tyrosine color value using a modified copper-phenol reagent procedure.[11] Tyrosine equivalents were read from a standard curve. Spontaneous serum proteolytic activity was determined by the above procedures by omitting SK from the digestion mixture. Appropriate controls were run to exclude any proteolysis due to the casein solution or to Varidase. Zero time values were determined by adding separately to TCA each reagent used in the test. One proteolytic unit was arbitrarily chosen as that activity necessary to cause an increase in color value equivalent to 50 μg of tyrosine during digestion under the conditions of this test.

Antitryptic Activity.—Serum trypsin inhibitor levels were determined by adding 1 ml. of 1:100 diluted serum to a trypsin-casein system (total volume of the digestion mixture was 3 ml.). A standard activity curve was prepared and all subsequent values based on this curve rather than on the micrograms of trypsin used since activity changes with storage period of trypsin solution (originally prepared at 1 mg. per milliliter in 0.0025 N HCl). Residual activity was determined from the standard curve as micrograms of trypsin necessary to release equivalent amounts of tyrosine-like fragments from casein. Activity differences between control trypsin-casein digestions and the same mixture containing serum represent the amount of trypsin inhibited.

Serum Protein Determination.—The Biuret method of Weichselbaum[12] was employed to estimate serum proteins. Whole human serum, the protein content of which had been determined by a micro-Kjeldahl method, was used as the standard protein solution.

RESULTS

Table I was prepared to show the type and duration of surgery for each of the patients.

Data presented in Table II indicate that SK-activated protease (SKP) usually drops below preoperative levels on the first day after surgery and remains so for variable periods. Fig. 1, which represents the arithmetical mean of all surgical cases presented for the first eight days following surgery, shows that the SKP rises during the first 12 hours and then drops below preoperative

*We wish to thank Mrs. Sally Hampton, Jefferson Davis Hospital, for obtaining these specimens.

†Hammersten quality; Nutritional Biochemicals Corp., Cleveland, Ohio.

‡Varidase; kindly supplied by Lederle Laboratories Division, American Cynamid Company, Pearl River, N. Y.

UNIVERSITY OF MICHIGAN LIBRARIES

TABLE IV. TOTAL SERUM

PATIENT	PREOPERATIVE DAYS 3	2	1	POSTOPERATIVE HOURS 3	6	9	12	DAYS 1	2	3	4	5	6	7	8
I. L.	7.05	7.10	7.15	7.25	---	7.25	7.10	7.90	5.70	6.25	6.70	6.00	6.25	6.10	6.70
M. G.	5.85	7.95	7.10	---	---	7.95	7.40	6.40	7.85	7.41	6.10	6.59	7.55	7.85	10.00
F. H.	7.05	7.15	7.70	---	7.15	7.85	---	6.70	7.15	7.15	6.55	6.85	---	6.70	
R. H.	7.05	7.40	---	---	---	---	---	6.85	8.70	6.25	4.85	7.15	7.55	6.55	7.05
V. B.	---	6.00	6.10	---	5.95	---	---	6.25	5.70	7.85	5.95	6.00	---	5.70	6.10
O. G.	---	---	6.00	---	6.70	---	---	7.55	7.15	7.10	5.40	5.70	6.70	5.70	---
S. R.	---	9.40	10.10	---	---	---	---	10.40	9.70	9.90	9.55	8.00	6.70	8.85	8.00
G. W.	8.10	7.85	8.70	8.25	6.55	7.70	---	7.25	8.40						
P. H.	---	7.25	6.25	7.55	7.40	---	7.05	6.25	6.85	6.00	6.70	7.40	7.05	7.25	8.85
T. F.	---	---	9.10	---	7.55	10.00	6.25	8.25	6.55	7.25	5.85	9.55	6.55	8.00	7.05
D. G.	---	---	8.10	7.25	7.70	7.25	---	7.70	7.85	8.10					
J. J.	---	---	7.05	8.40	---	---	---	7.70	8.95	7.70	7.75	7.25	7.05	---	7.70
A. D.	---	---	---	7.25	---	---	---	8.10	---	---	8.25				
R. W.	---	---	---	7.00	---	---	7.10	6.40							

levels from the first to the fifth day. Postoperative decreases in proteolytic activity were noted in every patient except G. W., on whom samples were not available after the second postoperative day. Sustained decreases in SKP were associated with those patients who underwent more extensive surgery (Fig. 2). Postoperative changes in Patient J. J. would seem to be within

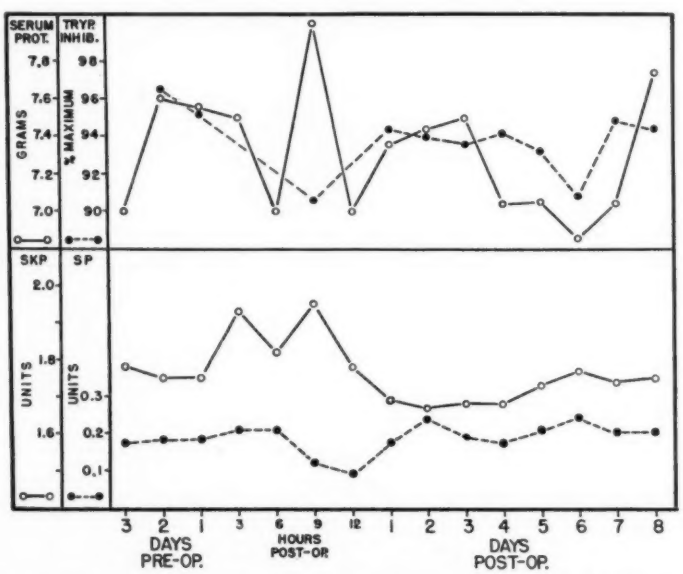


Fig. 1.—Arithmetical mean values for serum proteins, trypsin inhibitor, streptokinase-activated protease (SKP) and spontaneous protease. See Tables I, II, III, and IV for the data used to prepare this chart.

normal limits as evidenced by the preoperative level variations of several patients (Table II). Patient O. G. is of interest since an abdominal wall biopsy caused only mild decreases in SKP; however, after total body x-irradiation on the fourth postoperative day, marked fluctuations in SKP were observed. An attempt was made to confirm this finding concerning irradiation

PROTEINS (GRAMS PER CENT)

POSTOPERATIVE DAYS																
9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
7.95	8.95	7.40														
8.70	7.70	8.25	8.25	7.25	7.85	7.25	7.85									
5.95	6.15	6.25	5.70	6.70	7.05											
5.65	5.95	---	6.00	6.40	---	7.55	7.25	---	6.00	6.00	---	---	---	7.40	---	6.55
8.40	8.35	---	6.40	---	8.80	8.10	---	7.85	8.10	---	---	---	7.40	7.35	---	7.35
7.70																
6.40																

with Patient R. W., but samples were not available over a long enough period of time. Nevertheless, a marked SKP reduction was seen on the third post-irradiation day.

No correlations can be made between the preoperative SKP level and postoperative changes. Patient I. L. showed the greatest percentage drop in SKP (59 per cent).

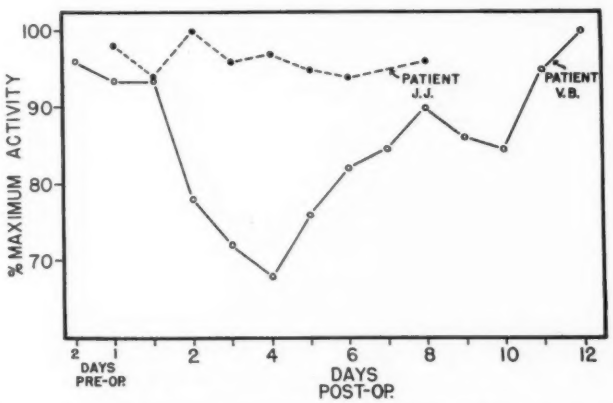


Fig. 2.—Streptokinase-activated protease of two surgical patients: J. J.—eye evisceration (30 minutes); V. B.—gastrectomy, splenectomy, and appendectomy (five hours).

Spontaneous protease activity (SP) was usually quite low (Table II). Although this activity ranges from 0 to 0.64 unit, the average values plotted in Fig. 1 indicate that postoperative changes are not marked, with the possible exception of a drop of approximately 0.1 unit at 9 and 12 hours after surgery. Such changes are of doubtful significance and no correlations between SP and the other values determined can be made.

Trypsin inhibitor measurements are tabulated in Table III. Changes were not marked, the maximum reduction observed was 74.5 per cent of maximum (Patient T. F., 12 hours postoperatively). Arithmetical means (Fig. 1) of the

trypsin inhibitor levels were never lower than 90.7 per cent of maximum. Comparison of Tables II and III does not show consistent correlations between trypsin inhibitor levels and either SKP or SP.

The results of total serum protein assays on the serum specimens are presented in Table IV, while mean values are plotted in Fig. 1. Variations observed cannot be directly correlated with any of the other assays made in this investigation. Numerous examples of this independent variation may be observed by an examination of Tables II, III, and IV.

No attempt has been made to correlate the measurements made with such variable factors as blood transfusions, drug administration, anesthesia, or postoperative course during this study.

DISCUSSION

The recent report by Astrup and Sterndorff[13] that normal human urine contains large amounts of plasminogen activator, as well as other reports of activators in the human body, makes the in vivo action of plasmin of probable significance. Scott and associates[5] have suggested that plasminemia causes abnormal postsurgical hemorrhage. Cliffton's study[1] using euglobulin revealed marked alterations in spontaneous proteolytic activity following trauma. In some cases, there was a tenfold rise during surgery. By the use of whole serum in this present study marked changes were not observed postoperatively. Spontaneous proteolytic activity was not observed to rise in the early postoperative periods, and other changes in this property previously cited are considered insignificant. The degree of rise in SKP early in the postoperative period is not marked, being on the average approximately 10 per cent.

It is of interest that in cases involving more extensive surgery more pronounced reductions in plasmin activity resulted. This could be due either to activation in vivo causing a depletion of the enzyme, or to an increase in inhibitor concentration. There are experimental evidences for each explanation, and only further studies can elucidate the contribution each makes to the over-all activity changes in the case of surgical patients. Increases in the $alpha_2$-globulin in the posttraumatic period[14] may contribute to the reduced proteolytic activity since this component of the serum proteins is rich in plasmin inhibitor.[15]

The postoperative changes reported herein may be correlated with other established posttraumatic events such as increased urinary excretion of 17-hydroxycorticoids[16] or other endocrinological and metabolic changes. However, no definite relationships can be established at this time with such events due to a paucity of information.

SUMMARY

In a study of thirteen surgical patients using whole serum it was found that usually there was a slight rise in the streptokinase-activated protease activity during the first twelve hours following surgery. The rise was followed by a fall in activity for variable periods of time, but usually beginning on the first or second postoperative day. The degree of variation generally

was related to the extent or duration of surgery. No correlations were observed between streptokinase-activated protease levels and spontaneous protease, serum protein, or antitrypsin levels.

We thank sincerely Dr. John M. Howard (formerly of the Department of Surgery, but now chairman of the Department of Surgery, Emory University), whose active interest and helpful suggestions made this study possible.

REFERENCES

1. Cliffton, E. E.: The Present Status of Therapeutic Use of Enzymes, Am. J. M. Sc. **228**: 568-585, 1954.
2. Müllertz, S., and Lassen, M.: An Activator System in Blood Indispensable for Formation of Plasmin by Streptokinase, Proc. Soc. Exper. Biol. & Med. **82**: 264-268, 1953.
3. Troll, W., and Sherry, S.: The Activation of Human Plasminogen by Streptokinase, J. Biol. Chem. **213**: 881-891, 1955.
4. Cliffton, E. E.: Variations in the Proteolytic and the Antiproteolytic Reactions of Serum: Effect of Disease, Trauma, X-ray, Anaphylactic Shock, ACTH, and Cortisone, J. LAB. & CLIN. MED. **39**: 105-121, 1952.
5. Scott, E. V. F., Matthews, W. F., Butterworth, C. E., and Frommeyer, W. B., Jr.: Abnormal Plasma Proteolytic Activity, Surg., Gynec. & Obst. **99**: 679-688, 1954.
6. Page Thomas, D. P., and Dingle, J. T. M.: Serum Antiplasmin and Plasmin in Rheumatoid Arthritis, Ann. Rheumat. Dis. **14**: 195-197, 1955.
7. Guest, M. M., Daly, B. M., Ware, A. G., and Seegers, W. H.: A Study of the Antifibrinolysin Activity in Human Plasmas During Pathological States, J. Clin. Invest. **27**: 793-794, 1948.
8. Shulman, N. R.: Studies on the Inhibition of Proteolytic Enzymes by Serum. II. Demonstration That Separate Proteolytic Inhibitors Exist in Serum; Their Distinctive Properties and the Specificity of Their Action, J. Exper. Med. **95**: 593-603, 1952.
9. Meyers, W. M., and Burdon, K. L.: Proetolytic Activity of Whole Human Serum Activated by Streptokinase, Arch. Biochem. and Biophys. **62**: 6-15, 1956.
10. Kunitz, M.: Crystalline Soybean Trypsin Inhibitor. II. General Properties, J. Gen. Physiol. **30**: 291-310, 1946.
11. Heidelberger, M., and MacPherson, C. F. C.: Quantitative Micro-Estimation of Antibodies in the Sera of Man and Other Animals, Science **97**: 405-6, 1943.
12. Weichselbaum, T. E.: Determination of Proteins in Blood Serum and Plasma, Am. J. Clin. Path. **16**: 40-49, 1946.
13. Astrup, T., and Sterndorff, I.: The Plasminogen Activator in Urine and the Urinary Trypsin Inhibitor, Scandinav. J. Clin. & Lab. Invest. **7**: 239-45, 1955.
14. Frawley, J. P., Howard, J. M., Artz, C. P., and Anderson, P.: Investigations of Serum Protein Changes in Combat Casualties, Arch. Surg. **71**: 605-11, 1955.
15. Jacobsson, K.: Studies on the Trypsin and Plasmin Inhibitors in Human Blood Serum, Scandinav. J. Clin. & Lab. Invest. (Suppl.) **7**: 57-102, 1955.
16. Moore, F. D., Steenburg, R. W., Ball, M. R., Wilson, G. M., and Myrden, J. A.: Studies in Surgical Endocrinology. I. The Urinary Excretion of 17-Hydroxy-corticoids, and Associated Metabolic Changes, in Cases of Soft Tissue Trauma of Varying Severity and in Bone Trauma, Ann. Surg. **141**: 145-174, 1955.

UNIVERSITY OF MICHIGAN LIBRARIES

SERUM PROTEIN TURNOVER IN MULTIPLE MYELOMA

SOLOMON A. BERSON, M.D., AND ROSALYN S. YALOW, PH.D.
BRONX, N. Y.

MULTIPLE myeloma is characterized by several unique abnormalities of protein metabolism. The hyperproteinemia[1] which frequently is present[2] is associated with a serum component migrating with the mobility of beta[2-4] or gamma globulins.[2, 4] Bence-Jones protein may be present in the urine in significant quantities whether or not anomalous serum proteins are evident[2, 5] and occasionally has been identified also in the serum.[6, 7]

It has been presumed that the anomalous proteins are products of synthesis of the myeloma plasma cells but the relationship between myeloma protein in the serum and Bence-Jones protein in the urine has been a subject of speculation. It has been suggested that Bence-Jones protein may represent a fragment of the serum myeloma protein,[5] but studies of the rate of incorporation of C^{13}- and N^{15}-labeled glycine into Bence-Jones protein, myeloma protein, and serum cryoglobulin[8, 9] led Putnam and Hardy to conclude that the evidence favored the independent origin of these proteins and was against any precursor-product relationship among them. More recently, from studies employing labeled DL glutamic acid, Putnam and associates[10] have considered the possibility that Bence-Jones protein may be a precursor or an abortive product of serum globulin synthesis.

Putnam and Hardy[8] observed a half-time for turnover of myeloma (β) globulin greater than that obtained for normal β globulins by London,[11] and suggested that the hyperproteinemia of multiple myeloma might be due to accumulation of the myeloma globulin by virtue of its slow turnover rather than to rapid synthesis. However, they felt that their data were insufficient to warrant a definite conclusion.

The present studies were designed to determine the rate of turnover of myeloma protein labeled with I^{131} and to evaluate in another way the possible derivation of Bence-Jones protein from serum myeloma protein. The investigation of protein distribution and metabolism by means of I^{131}-labeled proteins generally requires the isolation of highly purified protein preparations prior to labeling with I^{131}. However, if the protein of interest can be identified characteristically in a heterogeneous mixture of I^{131}-labeled proteins, isolation and purification are not prerequisite for certain purposes. In the present study, the exchangeable pools and turnover rates of "myeloma protein" and serum albumin were determined by I^{131}-labeling of the whole serum with identification of the labeled protein components by means of paper electrophoresis.

From the Radioisotope Service, Veterans Administration Hospital, Bronx 68, N. Y.
Received for publication Aug. 3, 1956.

METHODS

Two hospital patients with multiple myeloma served as subjects for this study. Patient P. A. showed an abnormal peak in the γ globulin region (''myeloma protein'')* on electrophoresis of the plasma (Fig. 1) but did not reveal evidence of proteinuria at any time. Patient M. H. excreted approximately 15 to 20 Gm. of Bence-Jones protein in the urine daily, although his plasma did not reveal any distinct abnormality on electrophoresis except for a slightly low albumin concentration (Fig. 1). A moderate trace of cryoglobulin was observed in the plasma but was not studied further. The urinary proteins concentrated in two zones, corresponding, respectively, to the regions between α_2 and β globulins and between β and γ globulins (Fig. 2). Electrophoretic heterogeneity of Bence-Jones protein from a single patient also has been reported by Putnam and Stelos.[12] Concentrations of total serum protein, determined by the Kjeldahl method, and of the various protein fractions, determined by planimetric measurement of the free electrophoresis patterns, are given in Table I.

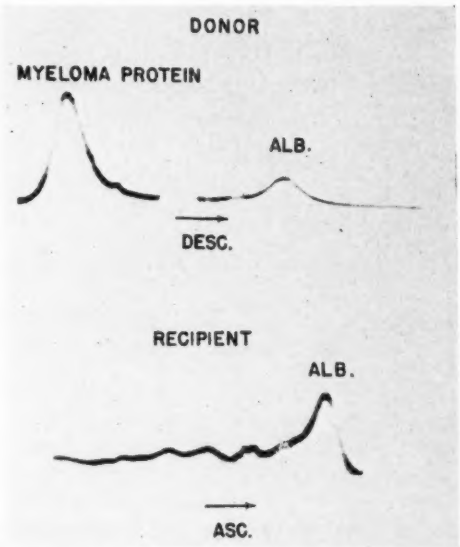


Fig. 1.—Electrophoretic patterns of the plasma in Donor Subject P. A., and Recipient Subject M. H., in Veronal buffer pH 8.6, 0.1 ionic strength.

TABLE I. PLASMA PROTEIN CONCENTRATIONS

DONOR P. A.	
Total protein	11.6 Gm./100 ml.
Albumin	2.28
α_1, α_2, β and fibrinogen	1.62
Myeloma protein	7.69
RECIPIENT M. H.	
Total protein	6.59 Gm./100 ml.
Albumin	3.58
α_1	0.59
α_2	0.82
β	0.72
Fibrinogen	0.51
γ	0.37

Serum was separated from the clotted blood of Patient P. A. and was trace labeled with I^{131} according to the method of Pressman and Eisen.[13] The iodinated serum was freed of nonprotein-bound I^{131} by dialysis in the cold and was then filtered through a

*Mobility, -1.0×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1}.

Seitz filter and checked for sterility by culture before use. Aliquots containing 117 μc I^{131} and 36 μc I^{131} were administered intravenously to the donor patient (P. A.) and to the recipient patient (M. H.), respectively. Blood samples were taken from both patients at frequent intervals after administration of the labeled serum and complete 24-hour urine collections were obtained for periods of 20 days. Assays of radioactivity in the administered doses, in plasma and in urine, were made in a well-type scintillation counter with a sensitivity of 1.00 $\times 10^6$ counts per minute (CPM) per μc I^{131} above a background of 200 CPM. Cold 10 per cent trichloracetic acid (TCA) was used to separate protein-bound from nonprotein-bound radioactivity. Nonradioactive plasma was added as carrier to the urine samples prior to addition of TCA in order to facilitate precipitation of any protein present.

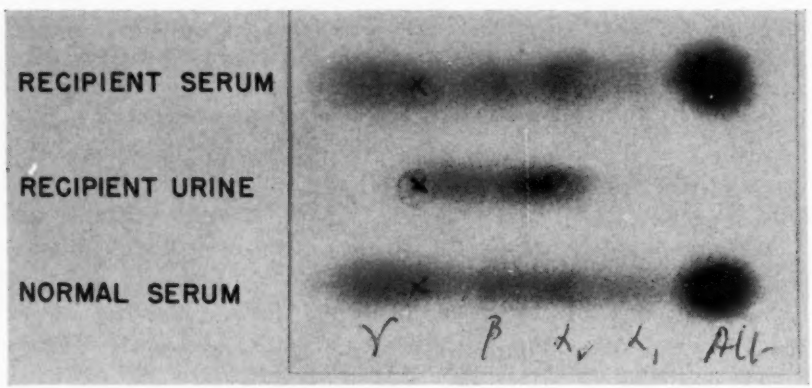


Fig. 2.—Paper electrophoretograms of serum and urine of Recipient Subject M. H., compared with serum of a normal subject. Six microliters of each sample were placed at points marked x.

The method used for paper electrophoresis has been described previously.[14] Phosphate buffer, ionic strength 0.1, pH 7.4, was employed. Fifty to two hundred microliters of plasma were applied to strips of Whatman No. 3 MM paper and after electrophoresis for 18 to 20 hours, the papers were dried and stained with naphthalene black. The strips from the early plasma specimens were assayed for radioactivity in a thin window flow gas counter employing an automatic strip scanner. The sensitivity of this arrangement was 0.60 $\times 10^6$ CPM per μc I^{131} over a background of 20 CPM. Radioactivities under myeloma protein and albumin peaks (Fig. 3, *A* and *B*) were determined by measurement of the respective areas with a planimeter. For the later points, where counting rates were low, 3 or 4 strips were run for each sample simultaneously. After the strips were stained, the myeloma protein and albumin zones of each were cut out, pooled, and counted in the well-type scintillation counter. The concentrations of radioactivity in the total plasma and in each of the two protein fractions were corrected for radioactive decay and plotted on semilog paper as a function of time.

The concentration-time curves were similar in general characteristics to those observed previously following administration of I^{131}-labeled serum albumin.[15, 16] The protein-I^{131} concentrations initially showed a rapid drop as distribution into extravascular compartments took place (Fig. 3, *A* and *B*). After distribution was essentially complete, an exponential decrease was observed which reflected metabolic turnover of the protein. The fractional rates of turnover of the proteins were obtained from the slopes of the terminal straight-line portions of the curves. The total radioactivities administered in each of the two fractions were divided by the respective ordinate intercepts of these straight lines to yield the apparent spaces of distribution. The total exchangeable pool for each was then calculated as the product of the respective serum protein concentration and apparent space of distribution. Since total and relative serum protein concentrations remained essentially unchanged throughout the course of observations, steady-state conditions were assumed and the rate of synthesis was taken as equal to the rate of degradation.

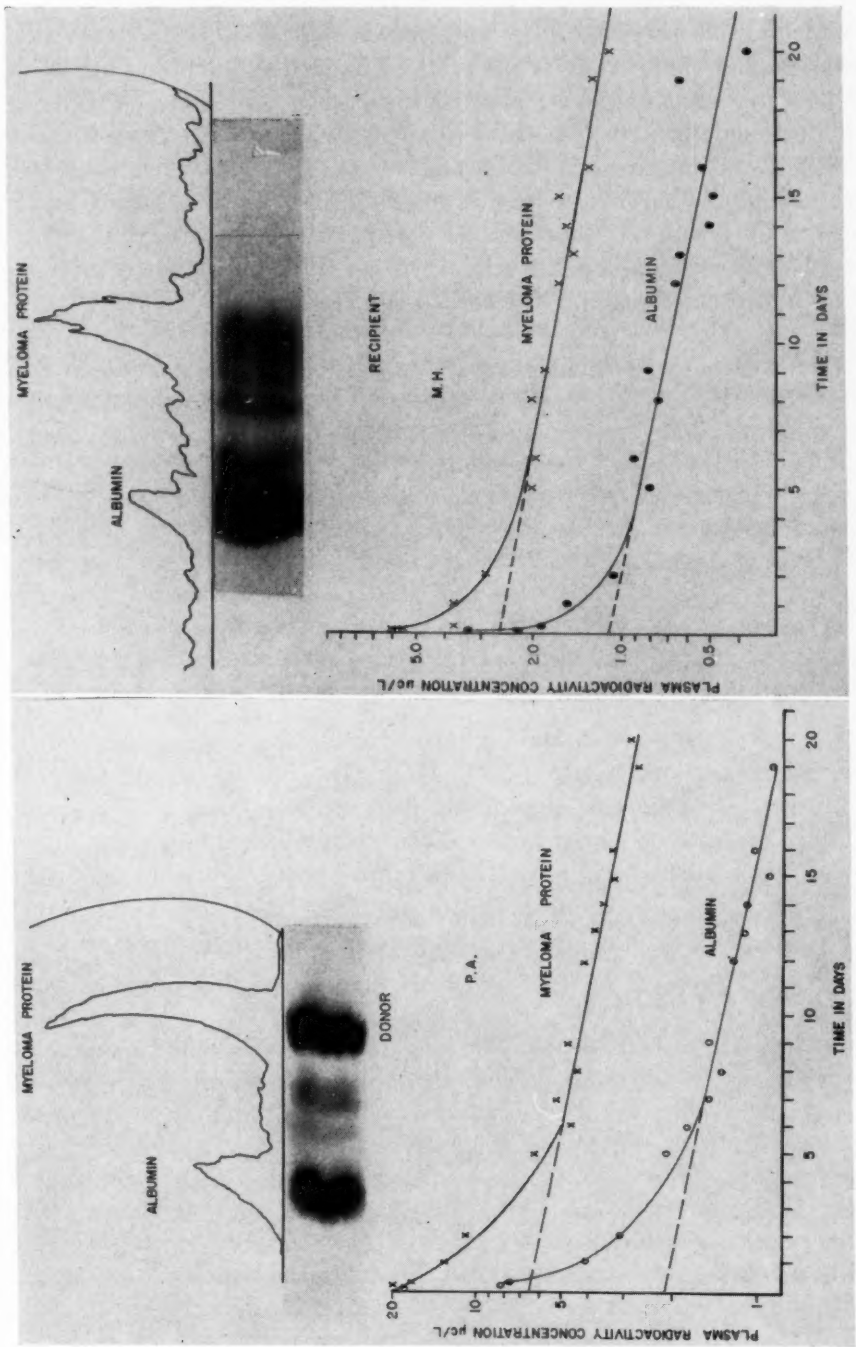


Fig. 3.—Sample paper radioelectrophoretograms of plasma and concentration-time curves of radioactivity under myeloma protein and albumin peaks in donor and recipient subjects.

RESULTS

Data related to the exchangeable protein pools and turnover rates are presented in Table II. The serum myeloma protein pool in the donor subject is about two and one-half times the total exchangeable albumin pool in either of the patients studied here or in normal subjects. Also, the daily turnover of serum myeloma protein (36 Gm. per day) is approximately two and one-half times that of serum albumin in normal subjects.[17] The fractional rates of turnover of total plasma radioactivity, serum albumin, and serum myeloma protein are all about the same and do not differ significantly in the two patients. They are also about the same as those which have been observed for albumin[16] and gamma globulin[18] in normal subjects.

TABLE II. DATA RELATED TO EXCHANGEABLE POOLS AND TURNOVER RATES OF SERUM PROTEIN

	EXCHANGEABLE POOL (GM.)	TURNOVER		DEGRADATION RATE (GM./DAY)
		HALF-TIME (DAY)	FRACTIONAL RATE (%/DAY)	
Donor P. A.				
Myeloma protein	820	15.5	4.5	36.8
Albumin	312	14	4.9	15.4
Total plasma radioactivity	—	14	4.9	—
Recipient M. H.				
Myeloma protein	—	17	4.1	—
Albumin	304	15	4.6	14.0
Total plasma radioactivity	—	18.5	3.8	—

The urine of the donor subject never exhibited any significant quantity of protein-bound radioactivity (Table III). The minute amounts of radioactivity which precipitated represent trapped nonprotein-bound I^{131}, since approximately the same fraction comes down even when I^{131} is added in vitro as iodine. Only about 5 per cent of the radioactivity in the urine of the recipient subject was precipitable, indicating that the major portion of the labeled serum had been subjected to metabolic degradation prior to excretion of the I^{131}.

TABLE III. TCA PRECIPITABLE RADIOACTIVITY IN URINE

DAY	DONOR P. A. (%)	RECIPIENT M. H. (%)
1	2.0	5.7
2	1.4	5.8
3	—	5.3
4	2.1	4.7
5	1.9	4.3
6	1.3	6.4
7	1.8	6.7
8	2.5	4.5
9	2.0	8.5

DISCUSSION

It has been observed frequently that Bence-Jones proteinuria may not be evident in subjects with high concentrations of myeloma globulin and also

that copious amounts of Bence-Jones protein may be excreted in the presence of normal serum protein patterns.[2, 5] These observations alone might lead one to question the concept that myeloma protein is a precursor of Bence-Jones protein. However, until recently the possibility could not be excluded that the absence of Bence-Jones proteinuria in subjects with high concentrations of serum myeloma globulin is due to an inability to degrade the myeloma protein to Bence-Jones protein; the absence of detectable myeloma globulin in the presence of Bence-Jones proteinuria could then be explained by the rapid conversion of the former to the latter. The present study was designed to test this possibility. The slow turnover rate of the transfused myeloma globulin makes it appear unlikely that the absence of detectable serum myeloma globulin in this subject was due to rapid transformation into Bence-Jones protein followed by excretion in the urine.

Furthermore, if the daily excretion of Bence-Jones protein (about 15 Gm. per day) had been derived entirely from the β and γ globulins of the serum, the renal plasma clearance of these proteins would have had to have been about 1,350 ml. per day, since the plasma concentration of $\beta + \gamma$ globulins was 1.1 Gm. per 100 ml. If the urinary protein were derived from a small fraction of these proteins the renal plasma clearance would have had to be even greater. Since the total plasma clearance of I^{131}-labeled serum was only about 400 ml. per day, and of this, 95 per cent was due to metabolic degradation and only 5 per cent due to direct protein loss in the urine, the derivation of Bence-Jones protein from serum myeloma protein is not supported by these studies. Moreover, the possibility cannot be excluded that the small amounts of protein-bound I^{131} present in the urine actually were due to slight albuminuria. Because of the small proportion of total urinary radioactivity in the protein fraction it was difficult to ascertain definitely which urinary protein was radioactive.

The findings reported here are consistent with previous observations of Putnam and Hardy on the absence of precursor-product relationship between serum myeloma globulin and Bence-Jones protein in subjects in whom both of these proteins were present. These workers have shown that following the feeding of C^{13}- or N^{15}-labeled glycine in acute experiments, the glycine of the excreted Bence-Jones protein closely approaches the calculated isotope concentration of the glycine pool of the body while the isotope concentration of the myeloma protein is initially considerably lower and is turned over more slowly. Since turnover of the glycine pool is extremely rapid[19, 20] these findings indicate a very brief life span of Bence-Jones protein within the body compared with that for myeloma protein, normal serum proteins, or tissue proteins. The marked variation, with respect to chemical and physical properties, of the myeloma globulins and Bence-Jones proteins found in different subjects as well as the gross heterogeneities of anomalous proteins observed occasionally in individual patients suggest that the disturbance in protein metabolism may not be limited to the production of one or two distinct species of abnormal protein. It rather appears as if a variety of anomalous serum

proteins, some perhaps similar to those produced normally in small amounts, may be elaborated in quantity by the myeloma tumors. Those proteins which are small enough to pass the glomerular filter readily appear as such in the urine shortly after they are released into the blood stream and consequently have an isotopic composition similar to that of the rapidly over-turning amino acid pool. This is characteristic of the Bence-Jones proteins which are generally in the molecular weight class of 30,000 to 45,000 and which have been shown to pass easily through the kidney of the cat, rabbit, and dog.[21] Those proteins in the molecular weight class of the serum albumins or in a greater weight class are retained by the kidney.[21] They therefore appear as abnormal proteins in the serum and are removed only by metabolic degradation at a relatively slow rate. These proteins consequently have a specific activity considerably lower than that of the amino acid pool and show a much slower turnover of isotope than does the Bence-Jones protein. There is no information on the rate of metabolic degradation of Bence-Jones protein or indeed whether it is subject to metabolic degradation at all.

The present study indicates that the high concentration of myeloma globulin in the serum is not attributable simply to accumulation resulting from a slow rate of degradation. On the contrary, the large daily synthesis of myeloma protein in the donor subject is in striking contrast to the rate of renewal of serum albumin[17] and γ globulin[18] observed in normal subjects. There was a half-time of 17 to 20 days for decrease in the N^{15} content of myeloma (β) globulin in the patient of Putnam and Hardy.[8] However, the total exchangeable pool size cannot be estimated by the N^{15} method and reutilization of N^{15} is probably responsible for some prolongation of the half-time beyond the true turnover rate. Barr and associates[22] estimated the rate of production of cryoglobulin at about 20 Gm. per day in a patient who showed no significant decrease in plasma concentration during a twelve-day period of significant blood loss from hemorrhage and phlebotomy. If the accelerated protein synthesis observed in the anomalous serum and urinary proteins of multiple myeloma reflects also the rate of turnover of the cell-fixed proteins of the plasma cells, it would then appear that the demand for amino acid substrates by the malignant tissues might well be sufficient to deprive other body tissues of adequate supplies. This would serve to aggravate the effect of whatever other factors may be involved in the production of the anemia, hypoalbuminemia, and wasting so frequently seen in this disease.

SUMMARY

1. The serum proteins of a patient with a high serum concentration of myeloma (γ) globulin but no Bence-Jones proteinuria were labeled with I^{131} and administered intravenously to this subject as well as to another patient who manifested marked Bence-Jones proteinuria but no anomalous serum proteins.

2. Analysis of paper radioelectrophoretograms of daily plasma samples permitted the determination of the exchangeable pools and turnover rates of serum myeloma protein and serum albumin in both patients.

3. The daily production of serum myeloma protein in the donor subject was about two and one-half times as great as that of serum albumin in normal subjects. The *fractional rate* of turnover of serum myeloma protein was not significantly different in the two subjects and about the same as that of serum albumin in normal subjects.

4. The excretion of protein-bound radioactivity in the recipient subject would have been insufficient to account for the Bence-Jones proteinuria had the latter been derived from serum myeloma protein.

5. This investigation does not support the concept of a precursor-product relationship between serum myeloma protein and Bence-Jones protein, respectively, but the possibility that Bence-Jones protein is a precursor or abortive product of serum myeloma globulin synthesis is not excluded.

We are indebted to Mrs. Melanie Knopf for the illustrations and to Miss Eve Spelke for secretarial assistance. We also wish to thank Mr. Manuel Villazon for the free electrophoresis determinations.

REFERENCES

1. Perlzweig, W. A., Delrue, G., and Geschickter, C.: Hyperproteinemia Associated With Multiple Myeloma: Report of an Unusual Case, J. A. M. A. **90:** 755, 1928.
2. Gutman, A. B., Moore, D. H., Gutman, E. B., McClellan, V., and Kabat, E. A.: Fractionation of Serum Proteins in Hyperproteinemia, With Special Reference to Multiple Myeloma, J. Clin. Invest. **20:** 765, 1941.
3. Longsworth, L. G., Shedlovsky, T., and MacInnes, D. A.: Electrophoretic Patterns of Normal and Pathological Human Blood Serum and Plasma, J. Exper. Med. **70:** 399, 1939.
4. Kekwick, R. A.: Serum Proteins in Multiple Myelomatosis, Biochem. J. **34:** 1248, 1940.
5. Rundles, R. W., Cooper, G. R., and Willet, R. W.: Multiple Myeloma. IV. Abnormal Serum Components and Bence-Jones Protein, J. Clin. Invest. **30:** 1125, 1951.
6. Kydd, D. M.: Bence-Jones Protein in Serum, J. Biol. Chem. **107:** 747, 1934.
7. Moore, D. H., Kabat, E. A., and Gutman, A. B.: Bence-Jones Proteinemia in Multiple Myeloma, J. Clin. Invest. **22:** 67, 1943.
8. Putnam, F. W., and Hardy, S.: Proteins in Multiple Myeloma. III. Origin of Bence-Jones Protein, J. Biol. Chem. **212:** 361, 1955.
9. Hardy, S., and Putnam, F. W.: Proteins in Multiple Myeloma. IV. Interaction With Metabolic Nitrogen, J. Biol. Chem. **212:** 371, 1955.
10. Putnam, F. W., Meyer, F., and Miyake, A.: Biosynthesis of Abnormal Proteins in Multiple Myeloma, Fed. Proc. **15:** 331, 1956.
11. London, I. M.: Studies on the Rates of Formation of Serum Proteins in Man. The Robert Gould Research Foundation, Inc., Cincinnati, Symposium on Nutrition **2:** 72, 1950.
12. Putnam, F. W., and Stelos, P.: Proteins in Multiple Myeloma. II. Bence-Jones Proteins, J. Biol. Chem. **203:** 347, 1953.
13. Pressman, D., and Eisen, H. H.: The Zone of Localization of Antibodies. V. An Attempt to Saturate Antibody-Binding Sites in Mouse Kidney, J. Immunol. **64:** 273, 1950.
14. Berson, S. A., Yalow, R. S., Bauman, A., Rothschild, M. A., and Newerly, K.: Insulin-I^{131} Metabolism in Human Subjects: Demonstration of Insulin Binding Globulin in the Circulation of Insulin Treated Subjects, J. Clin. Invest. **35:** 170, 1956.
15. Sterling, K.: The Turnover Rate of Serum Albumin in Man as Measured by I^{131} Tagged Albumin, J. Clin. Invest. **30:** 1228, 1951.
16. Berson, S. A., Yalow, R. S., Schreiber, S. S., and Post, J.: Tracer Experiments With I^{131} Labeled Human Serum Albumin: Distribution and Degradation Studies, J. Clin. Invest. **32:** 746, 1953.
17. Bauman, A., Rothschild, M. A., Yalow, R. S., and Berson, S. A.: Distribution and Metabolism of I^{131} Labeled Human Serum Albumin in Congestive Heart Failure With and Without Proteinuria, J. Clin. Invest. **34:** 1359, 1955.
18. Myant, N. B.: Observations on the Metabolism of Human Gamma Globulin Labelled by Radioactive Iodine, Clin. Sc. **11:** 191, 1952.

19. San Pietro, A., and Rittenberg, D.: A Study of the Rate of Protein Synthesis in Humans. I. The Measurement of the Urea Pool and Urea Space, J. Biol. Chem. **201**: 445, 1953.
20. San Pietro, A., and Rittenberg, D.: A Study of the Rate of Protein Synthesis in Humans. II. Measurement of the Metabolic Pool and the Rate of Protein Synthesis, J. Biol. Chem. **201**: 457, 1953.
21. Kerridge, P. D. T., and Bayliss, L. E.: The Physiology of Proteinuria and Its Clinical Significance, Lancet **2**: 785, 1932.
22. Barr, D. P., Reader, G. G., and Wheeler, C. H.: Cryoglobulinemia; Report of Two Cases With Discussion of Clinical Manifestations, Incidence and Significance, Ann. Int. Med. **32**: 6, 1950.

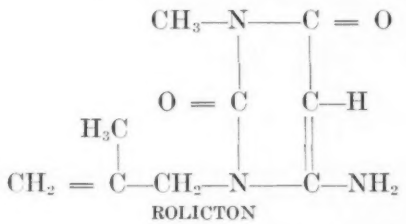
THE DIURETIC ACTIVITY OF A NEW PYRIMIDINEDIONE ADMINISTERED ORALLY TO NONEDEMATOUS ADULTS

MERVIN L. CLARK, M.D., AND JAMES A. HAGANS, M.D.*
OKLAHOMA CITY, OKLA.

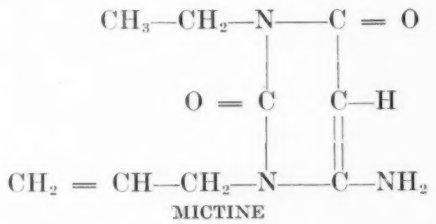
INTRODUCTION

THE difficulties encountered in diuretic evaluation utilizing edematous subjects have been emphasized by many investigators.[1, 2] That a diuretic effect can be detected in the nonedematous or normal subject has been demonstrated repeatedly,[1-5] and it would seem that such subjects provide a more stable and homogeneous base line for a comparative study. In the present study in nonedematous subjects observations were made on the diuretic effects of a new oral diuretic, Rolicton,† in comparison with an older oral diuretic, Mictine,‡ and were controlled by the administration of placebos.

Rolicton is one of the group of pyrimidinedione (aminouracil) compounds synthesized by Papesch and Schroeder[6] under the code number SC-3656. Its structural formula is similar to that of Mictine, differing only in the substituted groups on the uracil nucleus.



ROLICTON



MICTINE

Studies in animals have been carried out by Van Arman and co-workers[7] and Hambourger and associates[8, 9] and it was found that this compound appeared to have diuretic activity equal to Mictine, but markedly decreased emetic properties. Other toxic properties were minimal.

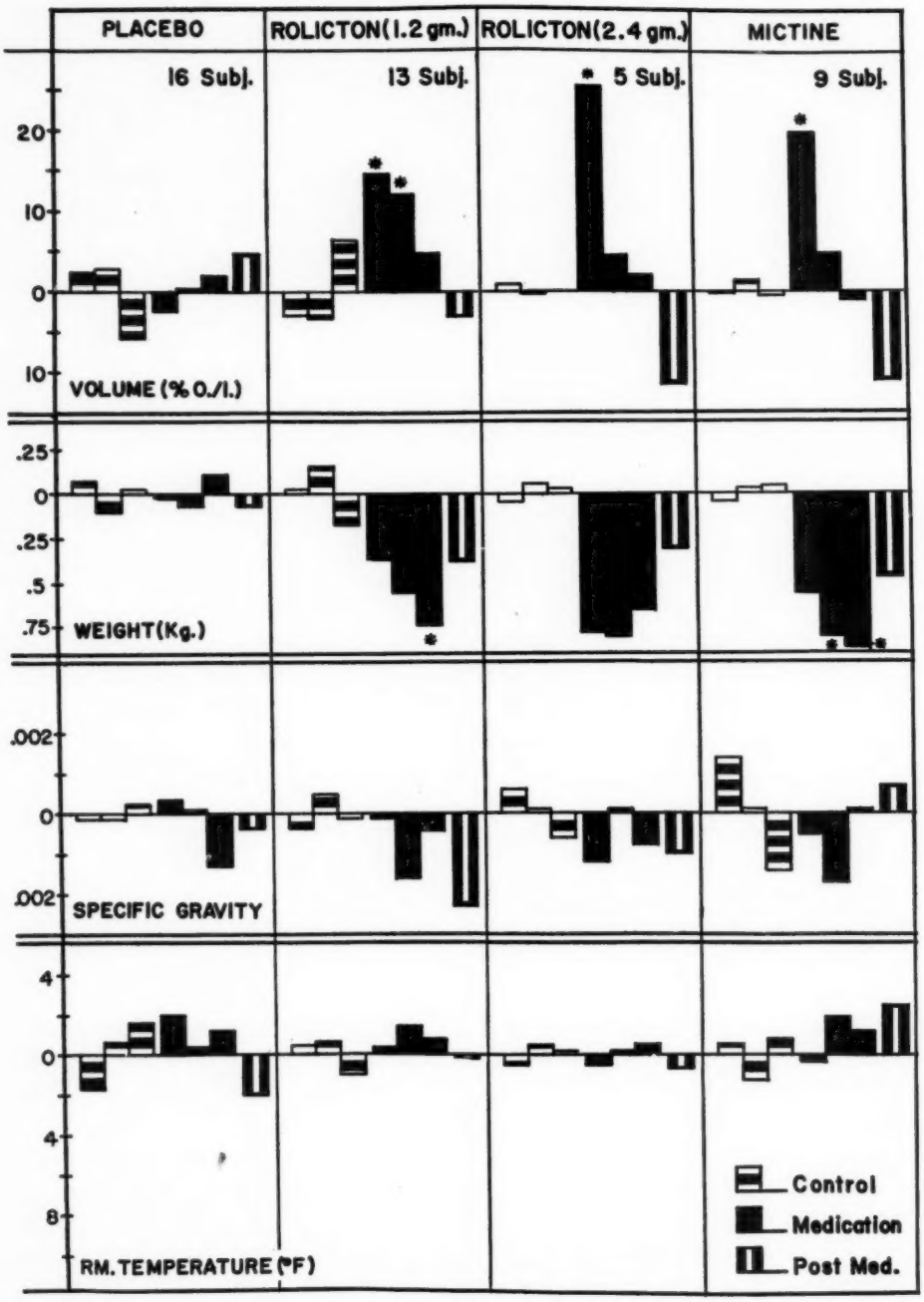
It has been reported previously that the mode of action of the pyrimidinedione compounds in man appears to be a specific inhibition of tubular reabsorption of sodium and chloride, not accompanied by an increase in glomerular filtration rate, or by any alteration in the factors regulating acid-base balance.[1, 4] The details of this mechanism are unknown.

From the Experimental Therapeutic Unit of the Department of Medicine, University of Oklahoma, and the Veterans Administration Hospital, Oklahoma City, Okla.
Received for publication June 7, 1956.

*Research Fellow, American Heart Association.

†G. D. Searle and Co., Brand of aminoisometradine (1-methallyl-3-methyl-6-aminotetrahydropyrimidinedione).

‡G. D. Searle and Co., Brand of aminometramide (1-allyl-3-ethyl-6-aminotetrahydropyrimidinedione).



*$p < .01$.

Fig. 1.—Daily mean variation of 24-hour urine volume, body weight, urine specific gravity, and room temperature for each medication group.

The 3 days of placebo administration (solid black bars) and the day following (vertically striped bar) showed no daily mean variations significantly different from the 3 days of control observations (horizontally striped bars). In contrast, the 3 days of medication in the other groups (solid black bars) showed significant increases in urine volume and decreases in body weight in the absence of significant variations in room temperature. No significant variations in 24-hour urine specific gravity were noted.

MATERIAL AND METHODS

Twenty-three male subjects ranging in age from 24 to 64 years were hospitalized on a special ward where careful attention could be accorded to food and water intake and to the collection of urine. None of the subjects showed any clinical evidence of cardiovascular or hepatic disease. Repeated urinalyses in each were normal and the blood nonprotein nitrogen was less than 35 mg. per cent. The subjects were placed on a regular hospital diet with no salt restriction. Each person drank the same amount of the same fluids each day, but among the subjects the total fluid intake ranged from 3,800 to 4,200 ml. per day. Each subject was studied over a period of seven days. Body weight was recorded at 8:00 A.M. daily and the 24-hour urine output was measured and specific gravity determined daily for each subject. Room temperature was recorded at 2:00 P.M. each day. Activity on the ward was not restricted. The first three days were regarded as the control period. During each of the succeeding three days each subject received one of the agents being tested without knowing what was being administered. The seventh day of observation served as a second control period.

Twenty-three subjects underwent a total of 43 test weeks, one to four tests per subject, usually on successive weeks. All medications were administered in divided doses three times a day with meals. Sixteen subjects received placebo, 13 received Rolicton 1.2 Gm. per day, 5 received Rolicton 2.4 Gm. per day, and 9 received Mictine 1.2 Gm. per day.

The data were recorded in the following manner:

The 24-hour urine volume was expressed as per cent of fluid intake (output/intake × 100). The mean 24-hour urine volume of the three day control period was calculated for each subject and the variation from this mean was determined for each of the seven days during the test week. The daily mean variation for each group receiving the same medication then was determined. Similar calculations were done for the body weight, specific gravity of the 24-hour urine, and the room temperature.

Significant deviations ($p < .01$ by t-test) from the mean control value for urine volume and body weight during the medication period were considered evidence of drug effect when room temperature variations were relatively small.

RESULTS

The data are recorded in Table I and expressed graphically in Fig. 1.

The changes noted during the administration of the placebo were essentially the same as those of the control period. A significant increase in urine volume and decrease in body weight were demonstrated with the administration of Rolicton (1.2 Gm. per day) and Mictine (1.2 Gm. per day).

Rolicton (2.4 Gm. per day) produced a marked increase in urine volume the first day of medication only, together with a substantial loss in body weight which was fairly well maintained over the three days of drug administration.

Specific gravity variations remained relatively small throughout the periods of observation and were not statistically significant. The mean variations in the 2:00 P.M. room temperature were minimal and considered to have no significant influence on the changes in urine volume and body weight.

Undesirable side effects occurred only in the Mictine treated group, where seven of the nine subjects complained of mild to moderate anorexia and nausea.

TABLE I. THE DAILY VARIATION OF 24-HOUR URINE VOLUME (OUTPUT/INTAKE × 100) AND BODY WEIGHT FROM THE MEAN CONTROL VALUE FOR EACH SUBJECT IN EACH MEDICATION GROUP

SUBJECT	MEAN CONTROL VOLUME (%)	DAILY VOLUME VARIATION FROM CONTROL MEAN (PER CENT) 1	2	3	4	5	6	7	MEAN CONTROL WEIGHT (KG.)	DAILY WEIGHT VARIATION FROM CONTROL MEAN (KG.) 1	2	3	4	5	6	7
PLACEBO																
1	76	+ 7	- 5	- 2	+15	- 6	+ 9	+ 7	74.91	-.14	+.09	+.09	0	+.23	+.91	+.55
2	86	+ 4	+ 5	- 9	-20	- 4	+11	+18	70.64	+.27	-.05	-.18	+.05	-.05	-.05	+.41
3	91	+ 3	+ 1	- 5	-11	+ 1	-27	- 4	86.68	+.14	-.09	-.09	+.14	+.36	+.27	-.18
4	91	0	- 3	+ 1	- 7	+ 4	- 2	- 5	55.59	0	0	0	-.14	0	0	+.23
5	85	+17	-15	- 2	-20	- 7	- 2	-20	62.18	-.14	-.14	+.32	+.55	+.55	+1.14	+.55
6	64	- 2	+12	- 9	-10	-11	-11	+14	65.09	-.18	-.09	+.27	+.36	+.50	+.36	+.59
7	82	0	+12	-13	+ 2	+ 2	+22	+16	80.05	+.09	-.14	+.09	-.05	+.32	+.09	-.05
8	89	+22	- 8	-15	+ 6	- 5	- 7	-15	80.95	+.32	-.59	+.32	+.09	+.09	-.27	-.05
9	81	- 3	+18	-15	+ 3	-18	-28	-15	108.55	0	-.45	+.45	-.59	-1.36	+.32	0
10	74	-16	-16	+28	- 9	+35	+ 6	- 8	62.45	-.05	+.18	-.18	-.05	-.86	+.50	-1.09
11	91	- 7	+13	- 7	+ 4	-12	+ 6	+ 3	64.45	+.09	0	-.14	-.14	+.09	+.09	+.09
12	74	- 3	+ 1	+ 2	+14	+13	+13	+19	66.09	-.05	+.18	-.18	+.05	-.41	-.73	-1.09
13	71	- 8	+25	-17	- 4	-13	+11	+19	81.41	+.41	-.27	-.14	-.59	-.05	-.05	-.59
14	51	- 6	+ 8	0	+ 1	+11	+ 1	+ 6	57.18	0	0	0	+.32	+.23	-.14	+.09
15	78	+11	0	-10	- 6	+ 2	+ 7	+ 6	87.18	+.55	-.14	-.36	-.36	-.68	-.82	-.91
16	76	+22	- 4	-19	+ 5	+12	+20	+32	61.14	-.09	-.09	+.14	-.09	0	+.23	+.23
$\bar{X}$	78.75	+2.57	+2.75	-5.75	-2.31	+0.25	+1.82	+4.56	72.78	+.08	-.10	+.03	-.03	-.07	+.12	-.08
ROLICTON (1.2 GM.)																
1	68	+ 3	+ 1	- 4	+22	+20	+19	+33	75.68	+.23	0	-.23	-.68	-.91	-1.14	-.91
2	71	-18	+ 4	+15	+33	+19	+26	+ 8	63.36	-.64	+1.18	-.50	-.18	-.50	-.86	+.05
3	93	+ 2	- 4	+ 2	+23	+16	+ 6	- 9	66.09	+.18	+.05	-.18	-.27	-.64	-.86	-.41
4	99	+ 5	-11	+ 5	+ 3	+ 5	+ 8	-13	57.50	-.32	0	+.36	+.36	+.23	+.23	+.14
5	106	+ 5	-11	+ 5	+ 5	- 9	- 7	- 9	85.77	+.14	+.14	-.32	-.77	-1.68	-1.55	-1.23
6	60	+11	- 2	- 8	-10	+ 2	-15	- 7	86.73	+.23	-.14	-.14	-.36	-.45	-.45	+.09
7	66	-14	- 4	+19	+36	+34	- 8	-14	63.77	+.32	+.32	-.59	-1.05	-.82	-1.05	-1.73
8	69	-26	- 1	+29	+23	+23	+ 2	- 8	65.23	0	+.23	-.23	-.91	-1.36	-1.14	-.91
9	80	0	+ 4	- 3	+17	+ 7	+ 5	+ 2	50.91	0	0	0	-.45	-.45	-.68	-.23
10	86	+ 8	- 4	- 4	+ 6	+ 5	+ 9	- 7	61.77	+.41	+.05	-.41	-.50	-.41	-.86	-.18
11	60	+ 4	- 8	+ 4	+14	+ 8	+11	+ 3	68.82	-.18	+.27	-.05	+.27	+.05	-.18	+.50
12	94	- 5	- 1	+ 6	0	+14	+12	- 6	62.64	-.23	+.09	+.09	+.23	+.32	-.36	-.14
13	81	-13	- 3	+17	+17	+11	- 4	-11	73.73	+.14	-.09	-.09	-.55	-.55	-.86	-.09
$\bar{X}$	79.46	-2.92	-2.50	+5.20	+14.54	+11.92	+4.92	-2.92	67.85	+.02	+.16	-.18	-.37	-.55	-.75	-.39

						ROLICTON (2.4 GM.)									
1	83	- 4	- 4	+ 8	+26	- 2	-11	-20	56.09	-.27	+.50	-.18	-.64	-2.00	-1.55	-1.55
2	78	+ 9	- 2	- 6	+11	+ 7	- 6	- 6	71.41	-.05	+.05	-.09	-.14	-.50	-.05	+.41
3	93	+10	- 6	- 4	+20	- 4	- 7	-29	67.45	-.05	+.05	+.05	-.86	-.95	-.95	-.18
4	86	+ 3	+ 8	-11	+28	+25	+ 6	- 1	65.45	0	-.23	+.23	-.32	-.09	-.45	-.45
5	48	-14	+ 2	+13	+42	- 4	+28	- 2	63.18	+.14	0	-.09	-2.05	-.55	-.23	+.23
$\overline{X}$	77.6	+0.8	-0.4	0	+25.4	+4.4	+2.0	-4.8	64.72	-.05	+.07	-.02	-.80	-.82	-.65	-.31
						MICTINE									
1	83	- 9	- 1	+10	+10	- 5	- 5	-12	55.73	-.05	+.09	-.05	-.27	-.59	-.36	-.36
2	67	+27	-13	-15	+ 9	0	+ 1	- 9	63.09	-.14	+.09	+.09	+.55	-.36	-.36	-.36
3	76	+ 9	- 1	- 7	+22	- 2	-19	-29	61.82	-.23	0	+.23	-1.14	-1.45	-1.45	-1.14
4	62	- 3	+ 3	+ 1	+42	+21	+17	-29	62.59	-.32	+.36	-.09	-.55	-.55	-1.45	-.55
5	78	- 7	+ 4	+ 2	+21	- 1	-10	-16	75.18	+.05	+.05	-.05	-.95	-1.41	-1.77	-1.41
6	76	+ 3	+ 8	-10	+24	+18	+21	- 4	63.45	+.09	-.27	+.18	-.59	+.18	-.05	+.86
7	84	-21	+15	+ 7	+22	- 8	+ 4	+ 7	67.64	+.45	0	-.45	-1.59	-1.77	-1.82	-1.50
8	84	+ 3	- 7	+ 5	+31	+12	-20	-14	82.05	-.23	0	+.23	-.77	-1.45	-.55	+.23
9	60	- 4	+ 3	+ 2	- 3	+ 6	0	+ 7	57.45	-.05	-.18	+.27	+.18	-.05	-.18	-.05
$\overline{X}$	74.4	-0.2	+1.3	-0.6	+19.8	+4.5	-1.2	-11.0	65.44	-.05	+.02	+.04	-.57	-.83	-.89	-.48

X = mean value.

DISCUSSION

The data on Mictine and Rolicton show evidence of diuretic action as reflected by an increase in urine volume and a loss of body weight. The significance of marked deviations from the control values is reinforced by the fact that the placebo induced no diuretic effect, the placebo data corresponding to that observed during the control periods. The maximum diuretic effect of Mictine and Rolicton was achieved on the first day. After withdrawal of the effective diuretic agent, "rebound phenomenon" was noted, characterized by diminished urine excretion and a gain in weight on the seventh day. Such a reaction lends indirect support to the evidence for the diuretic activity of the agents. The difference in side effects produced by the two agents was striking; Rolicton, even in the double dose, produced no discomfort whatever.

SUMMARY AND CONCLUSIONS

1. Rolicton, a new pyrimidinedione, administered to nonedematous subjects produces a significant diuresis manifested by increase in urine volume and loss of body weight compared to a control period and to a placebo. Its potency appears comparable to that of Mictine, but it is singularly free of the undesirable side effects (nausea, anorexia, abdominal cramps, and vomiting) which often limit the use of the latter drug.

2. The use of nonedematous or normal subjects for the evaluation of diuretic agents appears feasible. Should such results accurately reflect the therapeutic responses observed in edematous patients, the utilization of this more homogeneous group to evaluate the effectiveness of a new diuretic compound would then be justified and desirable.

REFERENCES

1. Kattus, A., Arrington, T. M., and Newman, E. V.: Clinical Observations on a New Oral Diuretic, 1-Propyl-3-Ethyl-6-Aminouracil, and Preliminary Studies on 1-Allyl-3-Ethyl-6-Aminouracil, Am. J. Med. **12**: 319-330, 1952.
2. Spencer, A. G., and Lloyd-Thomas, H. G.: Clinical Trial of a New Oral Diuretic, Brit. M. J. **1**: 957-960, 1953.
3. Citrin, Y., and Lyons, R. H.: Diuretic Potency of a New Organic Mercurial, CT-464 (Dicurin), New York J. Med. **54**: 1359-1361, 1954.
4. Assali, N. S., and Voskian, J.: Diuretic Effects of a Uracil Derivative (Mictine) in Normal and Toxemic Pregnancies, J. Appl. Physiol. **9**: 111-115, 1956.
5. Blumgart, H. L., Gilligan, D. R., Levy, R. C., Brown, M. G., and Volk, M. C.: Action of Diuretic Drugs. I. Action of Diuretics on Normal Persons, Arch. Int. Med. **54**: 40-81, 1934.
6. Papesch, V., and Schroeder, E. F.: Synthesis of 1-Mono and 1, 3-Di-Substituted 6-Aminouracils. Diuretic Activity, J. Org. Chem. **16**: 1879-90, 1951.
7. Van Arman, C. G., Dettelbach, H. R., and Kagawa, C. M.: Rolicton (SC-3656) Orally Active Diuretic of Low Toxicity, Division of Biological Research, G. D. Searle and Co. To be published.
8. Hambourger, W. E.: SC-3656: Five-Day Subacute Oral Toxicity Test in Dogs, Division of Biological Research, G. D. Searle and Co. To be published.
9. Hambourger, W. E., Calhoun, D. W., and Harris, T. W.: Chronic Oral Toxicity Studies with Rolicton in Rats and Dogs, Division of Biological Research, G. D. Searle and Co. To be published.

A STUDY OF THE EXCRETION OF Rb^{86}, K^{42}, AND K^{39} IN THERMAL SWEATING

G. S. BERENSON, M.D., AND G. E. BURCH, M.D.
NEW ORLEANS, LA.

ALTHOUGH considerable attention has been devoted to water and electrolyte composition of thermal sweat in normal and abnormal states, relatively few investigations have been concerned with kinetic phenomena of excretion of electrolytes in sweat. As an approach to this problem, the excretion of radioactive rubidium (Rb^{86})* and potassium (K^{42})* in thermal sweat was studied. Pharmacologic and biologic similarities between rubidium and potassium are known,[1-10] and although rubidium is not metabolically identical to potassium, Rb^{86} may be more suitable as a tracer of potassium in sweat than the short half-life K^{42} in experiments of long duration. Furthermore, the experiments were undertaken to determine the relative rates of excretion of Rb^{86} and K^{42} in thermal sweat and to elucidate further the mode of excretion of potassium and rubidium in sweat.

MATERIALS AND METHODS

Subjects.—Nine subjects free from congestive heart failure and endocrine disease were studied in these experiments. Their clinical data are summarized in Table I.

Areas of skin of the epigastrium and forearm were isolated with plastic chambers or cups, as described previously for the study in a comfortable atmosphere.[11] The subjects were then placed in a hot and humid atmosphere of 38° ± 1° C. and 95 ± 5 per cent relative humidity.

Administration of the Tracers.—The studies consisted of three groups of experiments:

Group I: Four subjects received Rb^{86} intravenously after the rate of sweating had become rapid after approximately fifty-six minutes' exposure to a hot and humid environment.

Group II: Three subjects received Rb^{86} intravenously prior to entry into the heated environment, two ten minutes prior to the experiment, and the third nine days previously.

Group III: Two subjects received intravenous Rb^{86} and K^{42} simultaneously after sweating had become rapid during exposure to a hot environment, as for Group I experiments.

Collection of Sweat.—Collections were made by three methods: (1) Sweat was pipetted from the chambers at five- to ten-minute intervals and sealed in glass ampules to avoid evaporation during storage. There was only a small error due to initial wetting of the surface of the skin and cup. (2) Sweat was scraped from the exposed surfaces of skin with the open edge of glass vials. (3) Sweat was absorbed by filter paper disks

From the Department of Medicine, Tulane University School of Medicine, and Charity Hospital of Louisiana at New Orleans.

Received for publication July 25, 1956.

Aided by a grant from the Public Health Service (H 143).

*Isotopes, Rb^{86} (half-life, 19.5 days) and K^{42} (half-life, 12.4 hours), were obtained from the Isotope Division, Oak Ridge National Laboratory.

placed on the anterior aspect of the exposed surface of the chest at intervals of fifteen seconds. Sweat obtained by the first technique alone was suitable for quantitative analysis.

Analyses.—For Group I experiments, the rate of sweating was expressed as milliliters per 10 cm.^{2} skin surface per 10 minutes, which represents the rate of excretion of water. The content of Na^{23}, Cl^{35}, K^{39}, Rb^{86}, and K^{42} in sweat collected within the plastic cups was also determined. Sweat collected by means of vials and paper disks was analyzed only for the presence of Rb^{86} and K^{42}. Total loss of body weight was determined on a clinical balance to ± 50 Gm.

The content of Na and K in the sweat was measured with a Beckman flame photometer, and the content of Cl was determined by the method of Schales and Schales.[12] Radioactive determinations were obtained with a thin-end mica window Geiger tube.[13] The activities of K^{42} and Rb^{86} were separated in the sweat by counting initially and then recounting after a lapse of over one hundred thirty hours, after correction for physical decay of both isotopes. Specific activities for K^{42} and Rb^{86} were expressed in relation to the concentration of K^{39} ([counts/ml./min.] / [meq./L.]).

Samples of blood taken before, during, and after the sweating procedure were also analyzed for the same electrolytes and radioactive isotopes. In one subject (No. 4), frequent blood samples were obtained after a single rapid intravenous injection of Rb^{86} in order that the concentration-time course of the tracer might be followed simultaneously in blood and sweat.

TABLE I. CLINICAL DATA

	SUBJECT NO.	AGE (YR.)	SEX	COLOR	DIAGNOSIS
Group I	1	63	M	C	Cirrhosis or metastatic carcinoma
	2	65	M	C	Chronic lymphatic leukemia
	3	65	M	C	Carcinoma of rectum
	4	67	M	C	Lobar pneumonia, convalescent
Group II	5	69	M	C	Peptic ulcer
	6	69	M	C	Possible peptic ulcer
	7	34	F	C	Tuberculous adenitis, arrested
	8	30	M	C	Metastatic carcinoma
	9	56	M	C	Possible Laennec's cirrhosis

RESULTS

The concentration-time courses of Na, Cl, and K in sweat and of the rates of sweating were essentially as reported previously.[11] Concentration of Na and Cl was relatively low initially and increased concordantly toward a maximal level but always remained hypotonic to serum. The concentration of K, which was much lower than that of Na and Cl, decreased and eventually reached a relatively constant level as sweating progressed but always remained higher or equal to the concentration of K in serum. The rate of sweating increased rapidly, achieved a fairly constant level, and remained for the duration of the experiment.

Time of Appearance of Rb^{86} and of K^{42} in Thermal Sweat.—In experiments for Groups I and II, the time required for the tracers to appear in collected sweat after sudden intravenous injection of the isotopes was fifteen to sixty seconds (Table II). More precise values, unfortunately, could not be obtained because of technical limitations. In Group II experiments, Rb^{86} was found in the

initial sample of sweat collected. These data indicate that Rb and K are transferred rapidly across the cells of the sweat glands. Table III shows the quantity and the estimation of the percentage of isotope administered that was excreted in sweat.

Curves of Concentration-Time Course.—The curves of the concentration-time course of electrolytes and radioactivity of Rb^{86} in sweat were essentially the same for the 4 subjects in Group I experiments (Figs. 1 and 2). The collected Rb^{86} appeared within fifteen seconds in sweat collected by means of filter paper disks placed on the skin. The Rb^{86} rapidly reached a maximal level in ap-

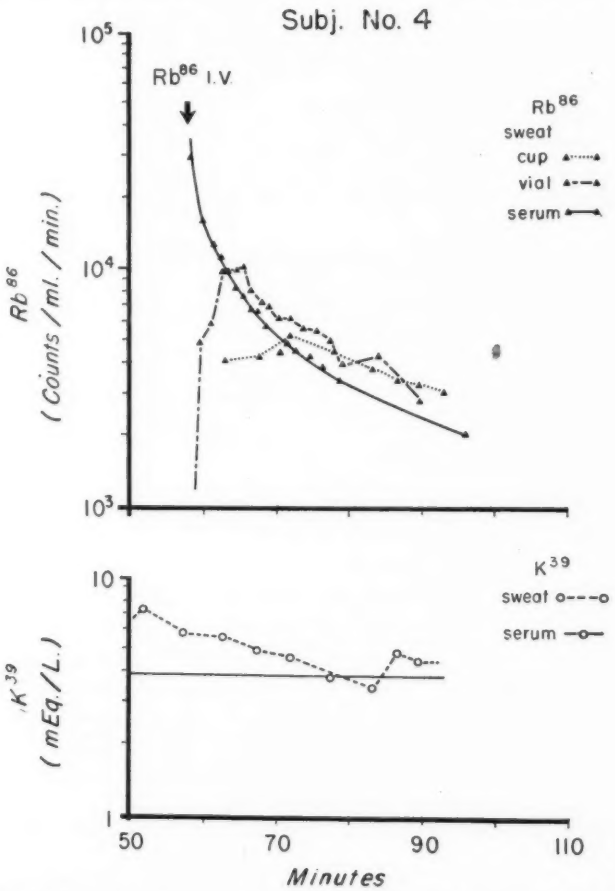


Fig. 1.—Concentration-time course of Rb^{86} and K^{39} in serum and thermal sweat after a single intravenous administration of the tracer in Subject 4, who was sweating profusely because of previous exposure for 56 minutes to a hot and humid atmosphere. "Cup" sweat refers to sweat collected in the plastic cups sealed to the skin; "vial" sweat refers to that sweat collected by scraping the skin with the open orifice of a small vial. The straight horizontal serum level shown for K^{39} was based on one determination within the limits shown and others beyond these limits.

proximately six minutes. The curves of concentration of Rb^{86} in the serum were similar to those described previously.[6] The concentration of Rb^{86} in whole blood and serum declined more rapidly and to levels lower than did that in the sweat. The concentration of potassium in serum changed little during the experiment, whereas the concentration of potassium in sweat declined slightly.

The curves of concentration-time course of Na, Cl, and K and the time course curves of "specific activities" of Rb^{86}/K^{39} of sweat and of "specific activities" of serum for a similar experiment, as well as the rate of sweating, are shown in Fig. 2. The ratio sweat "specific activity"/serum "specific activity" increased as sweating progressed and remained at approximately 1.3 during the experiment, the mean for the 4 subjects being 1.5 (range 1.3 to 2.0).

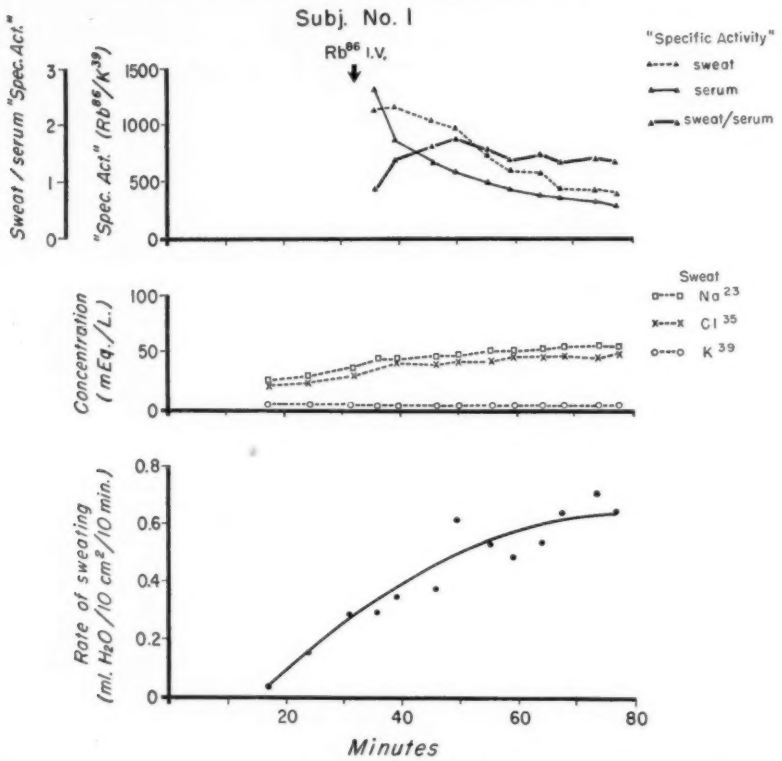


Fig. 2.—Time course of "specific activity" (Rb^{86}/K^{39}) of rubidium and sweat from abdominal skin following a single intravenous injection of the tracer in Subject 1, who had begun to sweat from previous exposure to a hot and humid environment. The time courses of concentration of Na^{23}, Cl^{35}, and K^{39} in the sweat and the rate of sweating are also shown.

TABLE II. TIME REQUIRED FOR DETECTION OF RADIOACTIVITY IN SWEAT AFTER INTRAVENOUS INJECTION*

	SUBJECT NO.	DURATION OF I.V. INJECTION (SEC.)	APPEARANCE TIME (MIN.:SEC.)
Group I (Rb^{86})	1	3-7	0:30
	2	3-7	1:00
	3	3-7	0:15
	4	3-7	0:15
Group II† (Rb^{86})	5	3-7	41:00
	6	5-10	28:00
	7	–	–
Group III (Rb^{86} and K^{42})	8	5-10	0:15
	9	40	0:30

*Measurements were obtained from sweat collected every 15 seconds onto paper disks.
†Isotope was injected prior to entry into the heated atmosphere; therefore, the times shown represent essentially the onset of sweating under the conditions of the experiment.

Time course curves for the Group II experiments were similar to those obtained for Group I. Subject 7, who received Rb^{86} nine days prior to the sweating period, demonstrated the same Rb^{86} "specific activity" for both sweat and serum as a result of sufficient time elapsing for Rb^{86} to approach equilibrium of distribution.

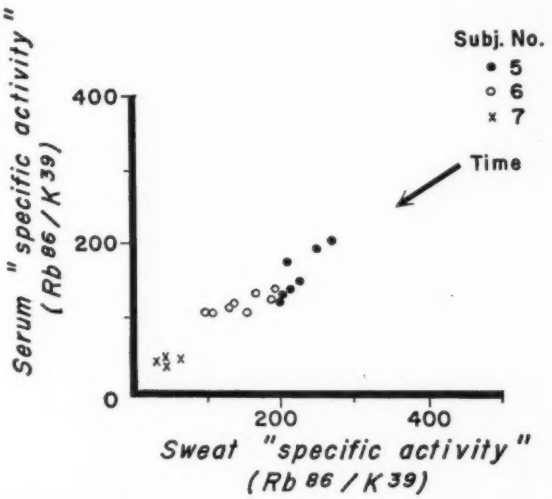


Fig. 3.—Relation of "specific activities" (Rb^{86}/K^{39}) of rubidium in sweat and serum of 3 subjects in Group II experiments after the concentration of Rb^{86} in serum and sweat had begun to regress at essentially a single exponential rate. The arrow merely indicates the direction of an imaginary line depicting time and, therefore, shows that as time progressed the values of "specific activity" declined.

Comment.—It is apparent in these studies that Rb^{86} quickly entered sweat from the blood, and the concentration of Rb^{86} in sweat rapidly reflected changes in concentration in the serum. Rb^{86} "specific activity," calculated on the basis of K^{39} and not rubidium, is not, of course, a true specific activity of Rb^{86}, but was obtained primarily for convenience of analysis and interpretation of the data. Fig. 3 summarizes the relation of the Rb^{86}/K^{39} "specific activities" for sweat and serum for the subjects of Group II. Such a close relation (Fig. 3) was not present immediately after intravenous administration of the Rb^{86} because of the rapid decline in serum "specific activity" initially. In spite of rapid transfer of Rb^{86} from blood to sweat, when sweating was rapid, temporal differences in Rb^{86} concentration in blood and sweat would be expected. Once the Rb^{86} concentration declined relatively slowly, such temporal differences became quantitatively less important. The deviation from unity of the ratio of Rb^{86} "specific activity" of sweat to serum, except in Subject 7, is a manifestation partly of the incomplete distribution of the isotope in the body; differences between rubidiokinetic and kaliokinetic phenomena must also account in part for the duration of the ratio from unity. These kinetic phenomena may not be significant, however, because Subject 7, who received the tracer nine days prior to the sweating, had a ratio of Rb^{86} "specific activity" of sweat to serum of unity.

Results of the Group III type of experiments, in which K^{42} and Rb^{86} were used simultaneously, are illustrated by Figs. 4 and 5 for one of the 2 subjects

TABLE III. APPROXIMATE AMOUNT OF ISOTOPE EXCRETED BY THERMAL SWEATING

	SUBJECT NO.	DOSAGE OF ISOTOPE (CTS./MIN. × 10^6)	EXPOSURE TO HEATED ENVIRONMENT (MIN.)	VOLUME SWEAT LOSS* (L.)	AV. CONCENTRATION IN SWEAT† (CTS./MIN./ML.)	SWEAT EXCRETION (% TOTAL DOSE)
Group I (Rb^{86})	1	186	77.5	2.04	2,808	3.1
	2	200	90.5	1.53	2,803	2.1
	3	200	85.0	1.30	4,326	2.8
	4	234	93.0	0.79	3,900	1.3
Group II (Rb^{86})	5	200	81.7	0.80	1,450	0.6
	6	100	68.3	0.91	511	0.5
	7	–	107.0	–	231	–
Group III (Rb^{86} and K^{42})	8	50 (of each isotope)	64.0	1.02	612K^{42} 389Rb^{86}	1.3
	9	50 (of each isotope)	91.8	0.85	2,272K^{42} 1,126Rb^{86}	3.9 1.9

*Estimated according to weight loss during period of sweating.
†From the abdomen.

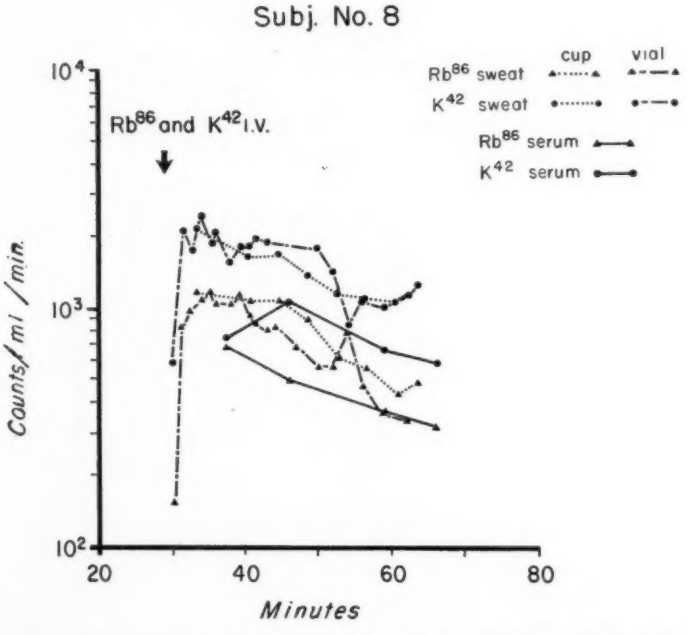


Fig. 4.—The concentration-time course of Rb^{86} and K^{42} in serum and sweat measured simultaneously for Subject 8 following a single, simultaneous injection of the two tracers. The subject had been exposed to the hot and humid environment for 29 minutes before administration of the tracers.

(Subject 8). The time of appearance of the radioactive substances (Fig. 4) resembled that shown in Fig. 1. Both tracers appeared on the surface of the skin rapidly after intravenous injection, increased to a maximal level of concentration, and gradually decreased. Although the curves of concentration-time course for Rb^{86} and K^{42} were similar in configuration, a quantitative difference was found. The concentration of K^{42} in serum and sweat and the K^{42} specific activity (K^{42}/K^{39}) were consistently higher than those for Rb^{86}. The

curves of time course of these various specific activities shown in Fig. 5 reveal these differences for Rb^{86} and K^{42} in greater detail. Fig. 6 shows further a comparison of specific activities of Rb^{86} to K^{42} in sweat and in serum. A linear

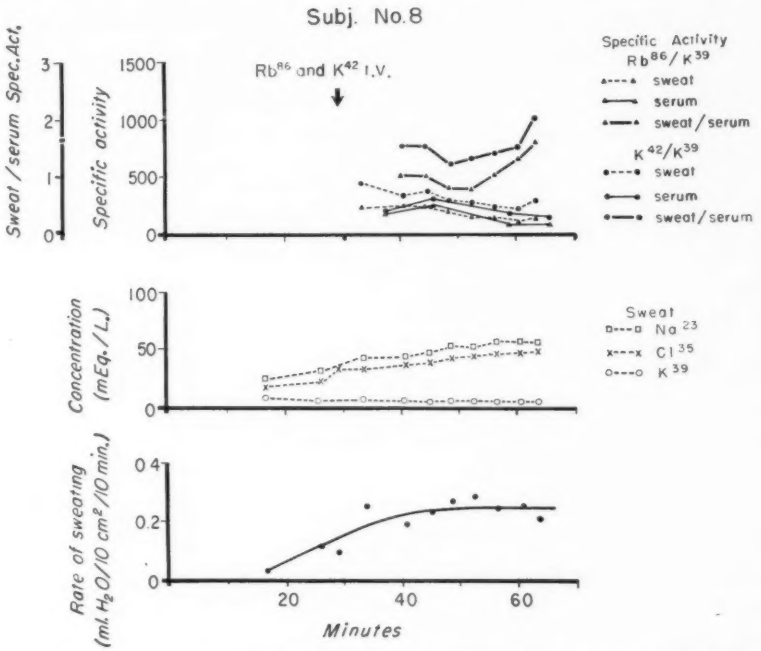


Fig. 5.—Curves of "specific activities" of rubidium, concentration of the electrolytes, and rate of sweating for Subject 8. Consult Fig. 4 for additional data on the same subject.

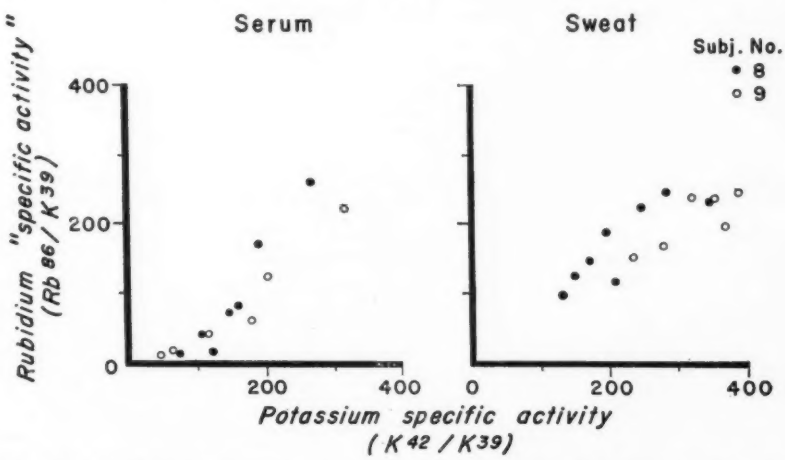


Fig. 6.—Relationship of "specific activity" of rubidium (Rb^{86}/K^{39}) to specific activity of potassium (K^{42}/K^{39}) in serum and sweat in Subjects 8 and 9.

relation between rubidium and potassium is suggested, with similar slopes for the specific activities in sweat and serum, indicating a constant and orderly transport of the two different elements from blood into sweat.

Although the general pattern of excretion of sweat of the two ions was remarkably alike, the consistently higher ratio noted for the specific activities of K^{42} than for Rb^{86} indicates a more rapid transport of K^{42} than Rb^{86}. This might be attributable in part to differences in dosages of the tracer, but the difference is probably a manifestation of kinetic difference between K and Rb, as shown by other observations.[6-10] Further studies are needed to establish in greater detail the mechanism for these differences.

DISCUSSION

Certain technical difficulties impose limitations in studies of sweating phenomena. Failure to obtain instantaneous quantitative collections of sweat from an individual gland at the surface of the parenchymal cell while simultaneously sampling the blood from which the sweat was produced renders interpretation of the relatively crude data of this and similar experiments difficult. The time required for the transfer of K or Rb from blood into sweat through the cells of the glands can only be estimated roughly from experiments. As mentioned previously, such interpretations are even more difficult, if not impossible, when the seral concentration of the tracer is changing, particularly if the change is rapid. Considerations of change in seral concentration are often neglected.

Calculations of the rate of clearance of blood serum of Rb^{86}, K^{39}, and K^{42} by the sweat glands ("sweat clearance") in units of milliliters per 10 cm.^{2} of skin surface per 10 minutes (Table IV) provide in different terms the rates of excretion of these isotopes. As in clearance studies for any organ during rapidly changing concentration of blood, calculations are subject to error and may be misleading, as evidenced by data obtained for the experiments in which K^{42} and Rb^{86} were administered simultaneously. For example, the data show that both K^{42} and Rb^{86} were cleared from the blood more rapidly than K^{39}.

TABLE IV. CLEARANCE OF RUBIDIUM AND POTASSIUM FROM SERUM BY SWEATING

	SUBJECT NO.	TIME FROM INJECTION OF ISOTOPE (ML./10 CM.^{2}/10 MIN.)					
		K^{39}			Rb^{86}		
		20 MIN.	30 MIN.	40 MIN.	20 MIN.	30 MIN.	40 MIN.
Group I	1	0.418	0.526	0.566	0.669	0.738	0.729
	2	0.274	0.375	0.465	0.487	0.618	0.714
	3	0.228	0.269	0.290	0.460	0.549	0.534
	4	0.324	0.395	–	0.416	0.418	–
Group II*	5	0.203	0.348	0.364	0.260	0.504	0.597
	6	0.223	0.258	0.262	0.252	0.281	0.276
	7	0.056	0.076	0.076	0.067	0.062	0.072

		K^{39} (ML./10 CM.^{2}/10 MIN.)		K^{42} (ML./10 CM.^{2}/10 MIN.)		Rb^{86} (ML./10 CM.^{2}/10 MIN.)	
		MEAN	RANGE	MEAN	RANGE	MEAN	RANGE
Group III†	8	0.284	0.277-0.292	0.445	0.397-0.518	0.440	0.325-0.576
	9	0.289	0.272-0.307	0.599	0.478-0.706	0.523	0.415-0.678

*Values were obtained after collection of first sample of sweat.

†Clearance values shown are means for the sweating period and are given for comparison of K^{39}, K^{42}, and Rb^{86}.

These experiments, like previous ones, reveal that stable K was excreted in higher concentration in sweat during the early periods of sweating than during

later ones, although the total excretion and clearance of K increased greatly with time. This observation seems to imply that, as sweating continued, the intracellular (sweat gland) source of K supplied proportionately decreasing amounts of K than are derived from extracellular fluid. This was also evident for Na, Cl, and water.

SUMMARY

Following an intravenous injection of Rb^{86} or K^{42} or both, Rb^{86} and K^{42} were detected in sweat within fifteen to sixty seconds, showing a rapid transfer from blood into sweat. Other observations suggest that the mechanism for the excretion of rubidium in the sweat closely resembles that for potassium, but further studies are necessary to define more precisely the relationship between these two elements. These experiments also show that, within limitations, Rb^{86} can be used to trace K^{39}. "Sweat clearances" for Rb^{86} and K^{42} in 2 subjects who received the isotopes simultaneously were similar in many respects. These studies further indicate that the excretion of K is derived primarily from a source of extracellular rather than intracellular fluid.

REFERENCES

1. Ringer, S.: An Investigation Regarding the Action of Rubidium and Calcium Salts Compared With the Action of Potassium Salts on the Ventricle of the Frog's Heart, J. Physiol. **4**: 370, 1883.
2. Mitchell, P. H., Wilson, J. W., and Stantan, R. E.: The Selective Absorption of K by Animal Cells; the Course of K Selection as Indicated by Absorption of Rubidium and Calcium, J. Gen. Physiol. **4**: 141, 1921.
3. Clark, A. J.: The Mode of Action of Potassium Upon Isolated Organs, J. Pharm. & Exper. Therap. **18**: 423, 1922.
4. Cowan, S. L.: The Action of K and Other Ions on the Injury Potential and Action Current in Maia Nerve, Proc. Roy. Soc., London, s.B. **115**: 216, 1934.
5. Follis, R. H.: Histological Effects in Rats From Adding Rubidium or Calcium to Diet Deficient Potassium, Am. J. Physiol. **138**: 246, 1943.
6. Burch, G. E., Threefoot, S. A., and Ray, C. T.: The Rate of Disappearance of Rb^{86} From the Plasma, the Biologic Decay Rates of Rb^{86}, and the Applicability of Rb^{86} as a Tracer of Potassium in Man With and Without Chronic Congestive Heart Failure, J. LAB. & CLIN. MED. **45**: 371-394, 1955.
7. Love, William D., and Burch, George E.: A Comparison of $Potassium^{42}$, $Rubidium^{86}$ and $Cesium^{134}$ as Tracers of Potassium in the Study of Cation Metabolism of Human Erythrocytes in Vitro, J. LAB. & CLIN. MED. **41**: 351-362, 1953.
8. Love, W. D., Romney, R., and Burch, G. E.: A Comparison of Potassium and Exchangeable Rubidium in the Heart and Other Organs of the Dog, Using Rb^{86}, Circulation Research **2**: 112, 1954.
9. Threefoot, S. A., Ray, C. T., and Burch, G. E.: Study of the Use of Rb^{86} as a Tracer for the Measurement of Rb^{86} and K^{39} Space and Mass in Intact Man With and Without Congestive Heart Failure, J. LAB. & CLIN. MED. **45**: 395-407, 1955.
10. Ray, C. T., Threefoot, S. A., and Burch, G. E.: The Excretion of Radiorubidium, Rb^{86}, Radiopotassium, K^{42}, and Potassium, Sodium, and Chloride by Man With and Without Congestive Heart Failure, J. LAB. & CLIN. MED. **45**: 408-430, 1955.
11. Berenson, G., and Burch, G.: A Study of the Sodium, Potassium and Chloride Content of Thermal Sweat Collected From Small Isolated Areas of Skin, J. LAB. & CLIN. MED. **42**: 58, 1953.
12. Schales, O., and Schales, S. S.: Determination of Chloride in Biological Fluids, J. Biol. Chem. **140**: 879, 1941.
13. Burch, G., Reaser, P. B., Ray, C. T., and Threefoot, S. A.: A Method of Preparing Biologic Fluids for Counting of Radioelements, J. LAB. & CLIN. MED. **35**: 626, 1950.

ABSORPTION AND EXCRETION OF SULFAMETHOXYPYRIDAZINE: A NEW, LONG-ACTING ANTIBACTERIAL SULFONAMIDE

ROGER L. NICHOLS, M.D., AND MAXWELL FINLAND, M.D.
BOSTON, MASS.

SULFAMETHOXYPYRIDAZINE* (3-sulfanilamido-6-methoxypyridazine) is a new antibacterial sulfonamide which, judging from experiments in dogs, rabbits, and mice, has the following properties: high solubility in urine, good absorption from the gastrointestinal tract, good penetration into the cerebrospinal fluid, and antibacterial activity equal to that of sulfadiazine.[1,2] These properties, and particularly the prolonged action, suggested that this agent might have a potential advantage over other sulfonamides should it also prove to be effective in prophylaxis, in the treatment of subacute or chronic infections, or in the suppression of chronic infections that are difficult to eradicate. The findings were therefore of sufficient interest to warrant exploration of this agent in humans.

Preliminary observations made in this laboratory[3] on normal men given a single dose of 4.0 gm. confirmed the previous notations of the qualities of good absorption and prolonged maintenance of high blood levels. In addition, it was shown that: (1) the drug in the blood, having penetrated little or not at all into the circulating blood cells was found in its entirety, or nearly that, in the plasma; (2) only a small proportion of the drug in the plasma was in the conjugated—presumably acetylated—form; (3) the plasma clearance of the latter averaged about 10 times that of the free form, and about one-half of the drug recovered in the urine was in a conjugated form, the solubility of which was considerably greater than that of the acetylated form in water.[1] The concentration of the free drug in the plasma between 5 and 12 hours after the dose of 4.0 Gm. was more than 20 mg. per 100 ml. in every subject, indicating that this dose was excessive and that about one-half of this amount or less would probably give optimum therapeutic levels from a single oral dose and possibly one-fourth of this amount or less might suffice to maintain such levels if given repeatedly at properly spaced intervals.

The present report deals with attempts to determine whether, in normal men, blood levels and urinary excretion of this sulfonamide could be influenced acutely by any of the following procedures: (1) high or low fluid intake; (2) oral administration of alkalis or of acidifying agents; or (3) administration of acetazolamide (Diamox) to obtain a diuresis with a highly alkaline urine.

From the Thorndike Memorial Laboratory and Second and Fourth (Harvard) Medical Services, Boston City Hospital, and the Department of Medicine, Harvard Medical School, Boston, Mass.

Aided by a grant from the National Institutes of Health.

Received for publication July 26, 1956.

*Generously provided by Lederle Laboratories Division, American Cyanamid Company, under the trademark Kynex.

A single dose of 3.0 Gm. of sulfamethoxypyridazine was chosen for these studies in the expectation that the prolonged maintenance of high levels in the plasma and the slow urinary excretion would permit the demonstration of any significantly positive acute effects. Also reported here are observations made on the concentrations of the drug in the plasma and urine of a few patients during repeated administration of doses of 1.0 Gm. at two-day intervals.

MATERIALS AND METHODS

The 6 subjects to whom a single dose of 3.0 Gm. was given were normal young men whose weight ranged from 68 to 90 kilograms and averaged 80 kilograms. The dose (6 tablets of 0.5 Gm. each) was given at 8 A.M. after an overnight fast, and food was withheld during the ensuing 7 hours; otherwise food was permitted ad libitum, as were also fluids, except as will be indicated in Subjects A and B in whom the effect of fluid intake was being evaluated during the first 2 days. The bladder was emptied of urine before the dose was given and complete collections of urine were then made for 10 days in each subject, and individual specimens were obtained at intervals thereafter until the drug was no longer detectable. Specimens of blood were taken in a dry oxalate mixture before the dose and at stated intervals during the ensuing 10 to 14 days; these intervals were frequent during the first 2 days, and blood and urine specimens were taken simultaneously to permit calculations of renal clearances of the drug from the plasma. The plasma was promptly separated from the specimens of blood and stored, along with aliquots of the measured amounts of urine, at −20° C. until the analyses were made. During the first 2 days part of each freshly voided specimen of urine was also examined microscopically for crystals and other formed elements and tested for sugar (with Benedict's reagent) and for albumin.

The doses of 1.0 Gm. every 48 hours were given to 7 hospitalized patients, 4 men and 3 women, who ranged in age from 17 to 66 years and in weight from 31 to 78 kilograms (average 53 kilograms). They were convalescent from a variety of illnesses but had no evidence of impaired renal function except for one patient, M.B., who had chronic pyelonephritis and a blood nonprotein nitrogen of 63 mg. per 100 ml. at the time of the first dose. Specimens of blood and of urine for drug levels were collected just before and 7 hours after nearly every dose, but no attempt was made to measure the total output of the drug in these patients. Urinalyses were also carried out frequently in these patients.

The concentrations of free and total sulfamethoxypyridazine in plasma and urine were determined by the method of Bratton and Marshall.[4] The pH of urine was determined on the freshly voided specimens with a Beckman pH meter. Blood sugar determinations were made in each of the subjects 5 and 7 hours after the dose and again at the same intervals after a similar overnight fast about 3 weeks after this study was completed; the method of Folin and Wu[5] was used for these determinations.*

RESULTS

Effect of Fluid Intake.—The plasma levels and data on urinary excretion of the drug during the first 48 hours in 2 subjects in whom fluids were restricted or forced for brief periods are charted in Fig. 1. Subject A had a water diuresis that began after several hours of forcing fluids and lasted through the second day; in subject B the diuresis was in progress when the dose was given and lasted through the first 12 hours. In these 2 subjects, there did not appear to be any clear correlation of the diuresis with any changes in the concentration of drug in the plasma, in its clearance from the plasma, nor in its rate of excretion into the urine. Subject B excreted a somewhat

*The chemical determinations were carried out by Miss Ellen J. Doyle and Miss Mary I. Kendrick.

greater proportion of the administered drug during each of the first 2 days, namely, about 25 per cent each day, as compared with Subject A who excreted 20 per cent during the first day and 15 per cent during the second. By the end of the sixth day, however, the differences were less striking; Subject B had excreted 82 per cent and Subject A 74 per cent of the dose.

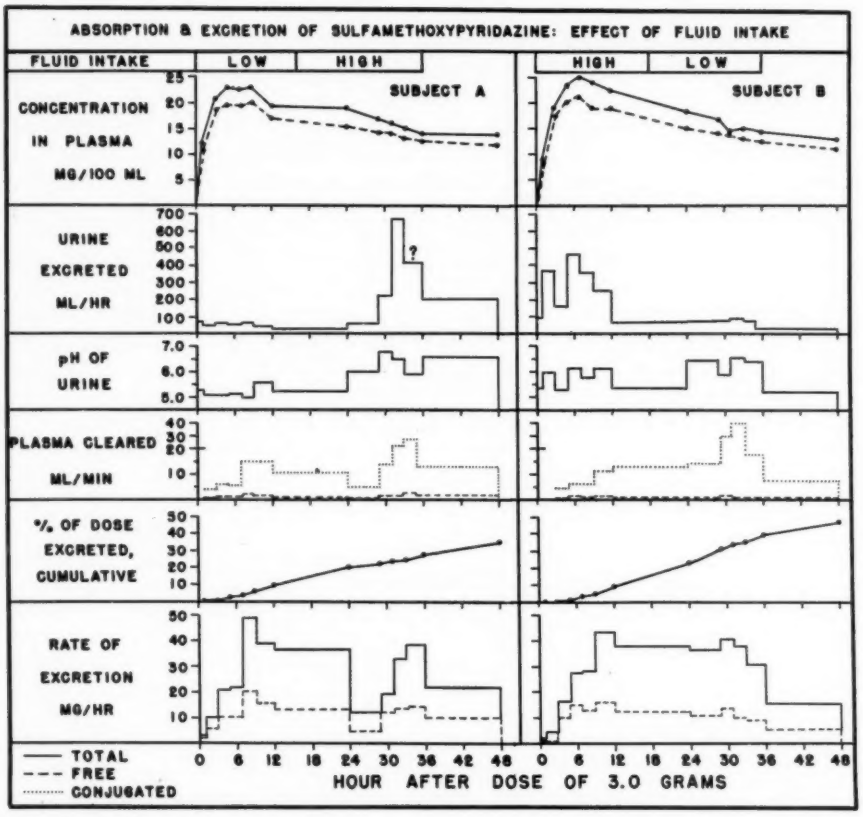


Fig. 1.—Subject A had a fluid intake of about 1,300 ml. for the 24-hour period ending 16 hours after the dose. During the ensuing 20 hours he took 3,500 ml. of fluid. In Subject B the procedure was reversed; he took 5,600 ml. during the 24-hour period ending 16 hours after the dose, and 2,100 ml. in the next 24 hours.

Effect of Acid and Alkali.—In this part of the study fluids were taken as desired, and attempts were made to produce an acid or an alkaline urine by the oral administration of acidifying or alkalinizing salts, respectively. The observations made during the first 48 hours in 3 studies on 2 subjects are shown graphically in Fig. 2. An alkaline urine was not achieved after the first attempt in Subject C but was achieved, for at least a few hours, after the second dose in this subject and also in Subject D while the alkalis were being given. During the administration of ammonium chloride and during most of the remaining period the pH of the urine was between 5.0 and 5.5 in both subjects.

There did not appear to be any correlation between the pH of the urine and the rate of change in the levels of sulfamethoxypyridazine in the plasma.

The rate of urine flow was also independent of its pH. The clearance of the acetylated drug appeared to be increased during the period when alkali was being administered and somewhat depressed when the urine was acid; the low plasma clearances of the free drug did not permit the demonstration of any clear changes. There was, however, some increase in the rate of excretion of both free and acetylated drug during the alkali administration as compared with the period when acid was being given, or at the other times when the urine was acid. Reflecting these differences, the slope of the curve for the cumulative excretion of the drug appears to be slightly steeper during the period when the pH of the urine was alkaline, at least in Subject D and during the second study in Subject C.

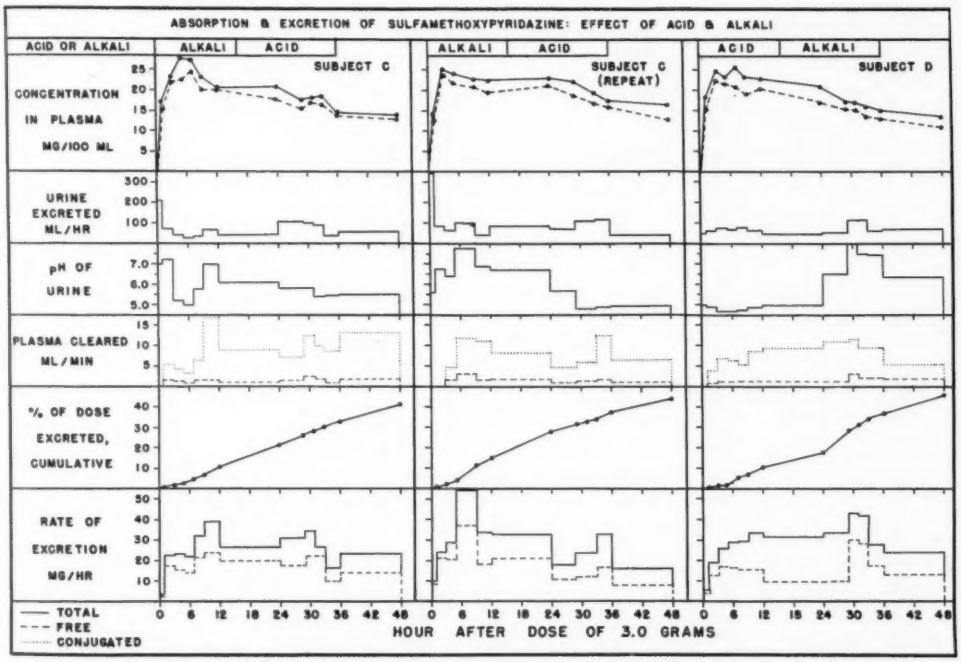


Fig. 2.—Subject C received 2 Gm. each of sodium bicarbonate and potassium citrate with the dose of sulfonamide and again 4 and 8 hours later; 12 hours after the dose he was given 3.0 Gm. ammonium chloride, followed by 1.5 Gm. 4, 12, and 16 hours later. Since an alkaline urine was not achieved during this attempt, the observations were repeated 24 days later with 2 additional doses of the alkalis. In Subject D, the procedure was reversed; he took ammonium chloride, 3.0 Gm., and 4 hours later 1.5 Gm. on the evening before the sulfonamide, 1.5 Gm. with the drug and a similar amount 4 hours later; 24 hours after the drug, he was given 2.0 Gm. each of sodium bicarbonate and potassium citrate and this was repeated 3 times during the next 8 hours.

Effect of Acetazolamide (Diamox).—Observations on absorption of the drug in 2 subjects who were given a dose of acetazolamide on the day after the dose of sulfamethoxypyridazine are charted in Fig. 3. In both of these subjects the administration of Diamox was followed by a prompt and significant water diuresis and a sharp rise in pH. Both of these changes were probably even more marked than would appear from the figure because the specimens of urine obtained during the 3 hours after the dose had been inadvertently mixed

with those obtained during the preceding 2 hours. The diuresis and the alkalinity of the urine were sustained for several hours, the latter for a period of over 21 hours. The plasma clearances of both free and acetylated drug, particularly of the latter were increased during the period of maximum diuresis and while the urine was alkaline. This was accompanied by a temporary lowering of the level of the drug in the plasma and reflected an increase in the rate of excretion of both free and total drug, which was particularly marked in Subject F.

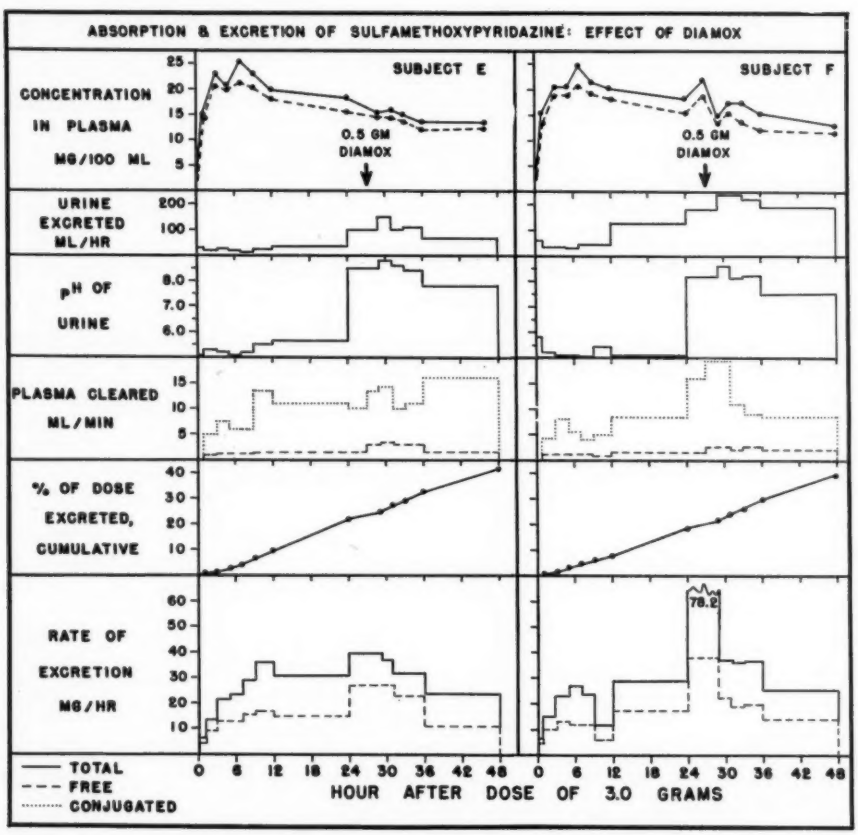


Fig. 3.—Subjects E and F were permitted fluids ad libitum; the former took about 1,600 ml. during the first 24 hours and 900 ml. during the second day after the dose, and the latter took about 2 L. each day. The acetazolamide was given orally 27 hours after the dose of sulfamethoxypyridazine.

Duration of Action of Single Dose of 3.0 Gm.—The concentrations of total sulfamethoxypyridazine in plasma after the 3 Gm. doses reached a peak in 3 to 9 hours (average 6.4 hours) averaged 25.7 mg. per cent and ranged from 23.0 to 27.8; the corresponding average peak levels of free drug averaged 21.8 mg. per 100 ml. and ranged from 20.2 to 23.8. After 24 hours, the average values declined to 20.0 and 16.9 mg. per cent for total and free drug, respectively, and the corresponding average values at 48 hours were 13.9 and 12.0 mg.

per 100 ml.; thereafter the decline was steady for another 8 days as shown in Fig. 4. The average amount of drug determined as conjugated at each interval was usually about 10 to 15 per cent of the total.

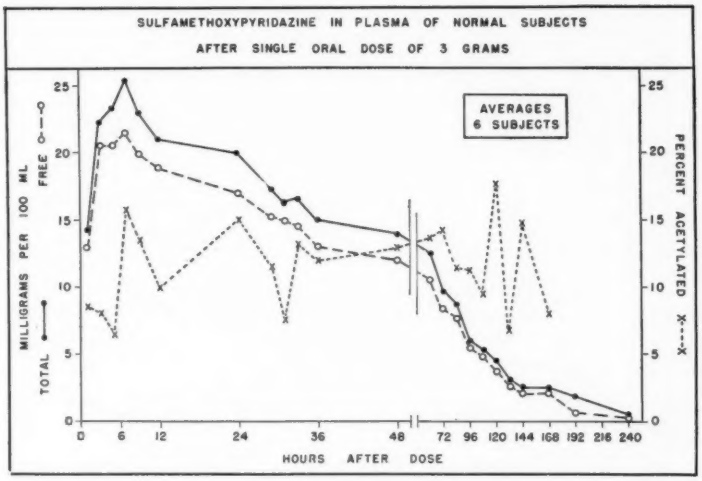

SULFAMETHOXYPYRIDAZINE IN PLASMA OF NORMAL SUBJECTS
AFTER SINGLE ORAL DOSE OF 3 GRAMS
AVERAGES
6 SUBJECTS
MILLIGRAMS PER 100 ML
TOTAL
FREE
PERCENT ACETYLATED
HOURS AFTER DOSE
0 6 12 24 36 48 72 96 120 144 168 192 216 240
0 5 10 15 20 25

Fig. 4.

Concentrations of more than 1.0 mg. per 100 ml. were still demonstrated in the plasma of all 6 subjects 8 days after the 3.0 Gm. dose of sulfamethoxypyridazine, but only in Subject A were they found on the tenth day. Similar concentrations were found in the urine of all subjects on the tenth day, in 4 of 5 subjects whose urine was tested on the twelfth day, and in only 1 of 3 tested on the fourteenth day. The entire range of concentrations, as well as the average values during the successive periods attained in the urine is shown in the middle portion of Fig. 5. These values were obviously dependent on the volume of urine excreted, which fluctuated very widely. The values for the *rates* of excretion, as may be expected, showed considerably less variations at the different periods than did the concentrations of drug in the urine at the same periods; this may be seen from the upper portions of the same figure.

The greatest total amount recovered from the urine was 96 per cent of the administered dose in Subject E; the lowest recovery was 82 per cent of the dose in Subject A, and the average was 85 per cent for all 6 subjects. During most of the period of excretion of the drug, between 50 and 65 per cent was recovered from the urine in the conjugated form, the proportion being lowest during the first day and highest during the fourth to the sixth days. The total amount of drug recovered in the free form averaged 38 per cent of the administered dose and ranged from 32 per cent in Subject A to 51 per cent in Subject C. Of the total amounts excreted, the proportion found in the conjugated form averaged 57 per cent, and varied from 46 per cent in Subject C to 72 per cent in Subject B. These findings are depicted in the lowest panel of Fig. 5.

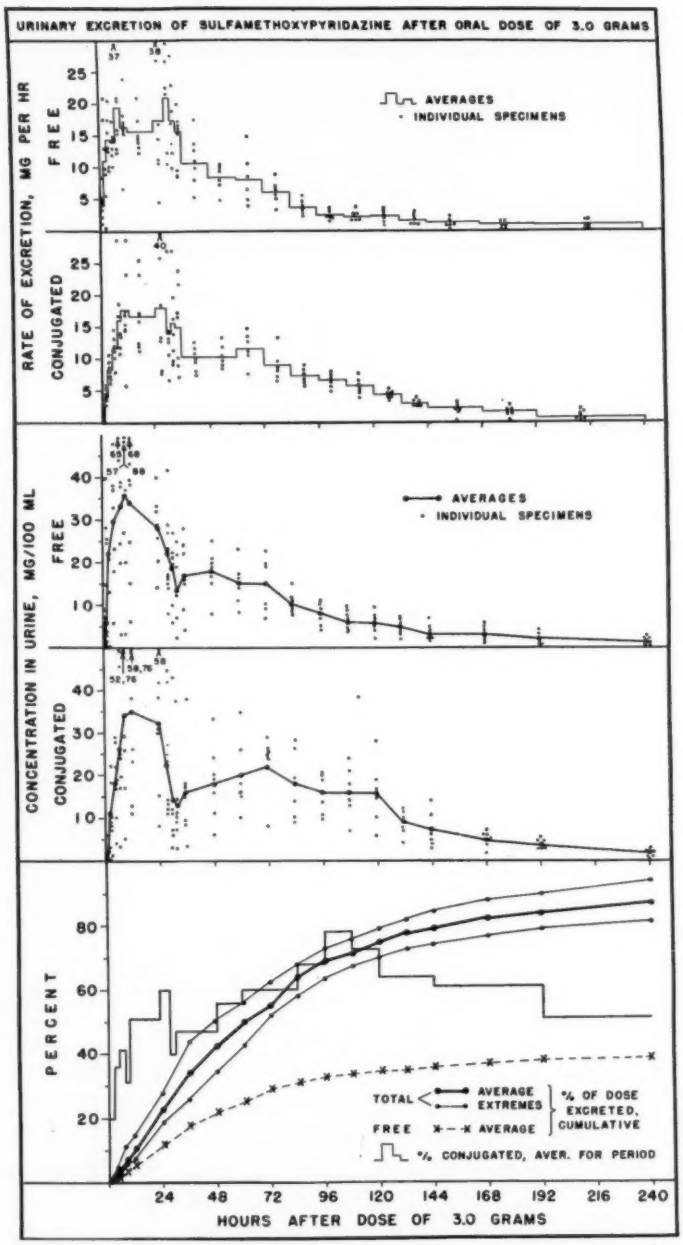


Fig. 5.—The 2 uppermost panels show the individual and average *rates of urinary excretion* of the free and conjugated drug at different intervals after the dose. The next 2 panels show the *concentrations* of free and conjugated drug in the individual specimens of urine, and the averages for the specimens collected at successive intervals after the dose. The lowest panel depicts the extreme and the mean values for the cumulative amounts of total sulfonamide and also the average of the free portion excreted in the urine; the average per cent of drug recovered in the acetylated form at successive intervals after the dose are also shown here.

Repeated Doses of 1.0 Gm. Every 48 Hours.—From the practical therapeutic point of view, the dose of 4.0 Gm. used in the preliminary study and the 3.0 Gm. used in the study just presented were obviously excessive. Since the antibacterial activity of sulfamethoxypyridazine, weight for weight, is quite similar to that of sulfadiazine,[1, 2] and since plasma levels of about 10 mg. per 100 ml. are generally considered to be optimal for the latter sulfonamide, it appeared likely from the foregoing data that such levels of sulfamethoxypyridazine could be approximated by administration of 1.0 Gm. every other day. To determine whether this was so, 7 patients varying in age and weight and having or convalescing from a variety of illnesses were placed on this dosage regimen. Specimens of blood and urine were obtained from each of these patients at 7 and 48 hours after each dose as far as was feasible.

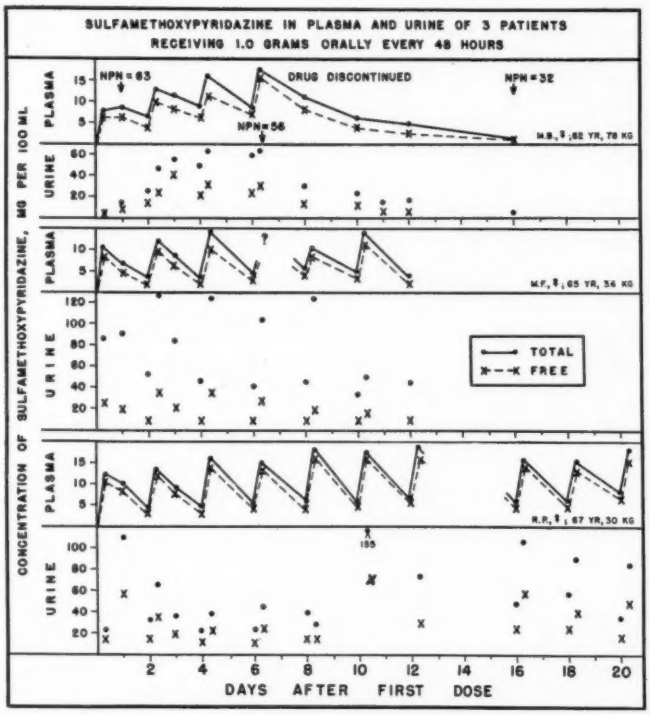


Fig. 6.—Findings in 3 patients illustrating the extreme values and the fluctuations observed. Patient MB had rheumatic heart disease and pyelonephritis with evidence of impaired renal function. Patients MF and RP were markedly underweight.

In one patient (M. B., Fig. 6) the concentrations in the plasma 7 and 48 hours after each dose rose progressively, reaching a level of 17.6 mg. total and 15.4 mg. free drug per 100 ml. 7 hours after the fourth dose. No further doses were given and the concentrations of drug in the plasma fell progressively to barely demonstrable levels in the ensuing 10 days. The concentrations of drug in the urine also increased and declined along with the plasma levels. During the administration of the drug, and while it was being excreted, the nonprotein nitrogen (NPN) dropped progressively from 63 to 32 mg. per 100 ml. by the

sixteenth day; however, the patient's renal disease subsequently relapsed and she had a progressive rise in NPN over the following few weeks. In 2 other patients (M. F., and R. P., also shown in Fig. 6) there was also a slight increase in the maximum plasma concentration after each of the first 3 or 4 doses, after which the levels were stabilized. The concentrations in the urine of these 3 patients fluctuated widely, as may be expected, since the volume of urine and the rate at which it was excreted varied considerably at different times.

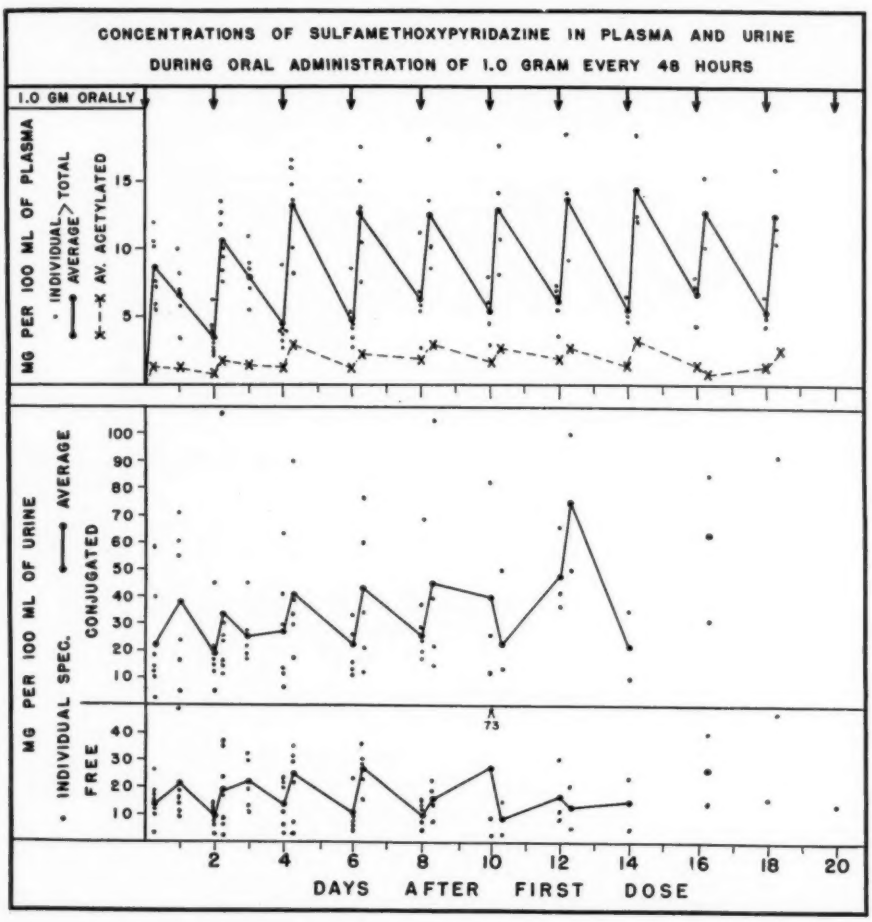


Fig. 7.—Individual and mean values for 7 patients.

The mean values for the 7 patients (Fig. 7) also reflect the gradually increasing plasma levels achieved in the blood after the first few doses. They indicate that, on the dosage regimen of 1.0 Gm. every 48 hours, the average concentrations of sulfamethoxypyridazine in the plasma fluctuated from a low of 5 to a high of about 12.5 mg. per 100 ml. Of this amount, between 1 and 3 mg. and an average of less than 2 mg. per 100 ml. was in the acetylated form.

Effect on Blood Sugar.—Fasting blood sugar determinations were made 5 and 7 hours after each of the doses of 3.0 Gm., and again in the same individuals

after a similar fasting period about 4 weeks after the dose. No evidence of hypoglycemia was elicited in any of these subjects, at least at about the time when they had achieved peak concentrations of the drug in their plasma.

Untoward Effects.—As in the preliminary study when 4.0 gm. were used, the only untoward effect observed after the 3.0 Gm. dose was administered was headache. This was experienced after 5 of the 7 doses and by 5 of the 6 subjects, Subject C having no symptoms after the first dose. The headache was moderate to severe and was chiefly frontal; it began in different subjects from 3 to 8 hours after the dose and lasted for several hours. It was generally relieved by lying down and was aggravated by sudden motion of the head. Three of the subjects took 0.6 Gm. of aspirin and obtained relief in each instance. One subject experienced lightheadedness and giddiness before the onset of the headache and again on the following morning. There were no gastrointestinal or other symptoms. Crystalluria or other urinary abnormalities could not be demonstrated. None of the patients who received repeated doses of 1.0 Gm. every other day experienced headache, and no other untoward effects were elicited in these patients.

DISCUSSION

The findings indicate that in humans sulfamethoxypyridazine is almost completely absorbed and excreted into the urine after oral administration. High levels of this drug are maintained for prolonged periods after ingestion of single doses of 4.0 or 3.0 Gm.; these levels, at their peaks, represent 2 or more times the concentrations that are generally desired and may be related to the headaches experienced by the majority of the subjects who ingested such doses. On the other hand, the continuous administrations of 1.0 Gm. every other day, which was not accompanied by any headache or other untoward effects, yielded plasma concentrations which would generally be considered optimum; these were comparable to the levels observed in the majority of patients treated with sulfadiazine in doses of 1.0 Gm. every 4 or 6 hours.[6] The concentrations of the drug in the urine, however, were much lower during treatment with sulfamethoxypyridazine.

The prolonged residence of the drug in the body with its persistence in high levels in the plasma prompted the attempts to determine whether its excretion could be hastened by an increase in the output of urine, by the excretion of an alkaline urine, or by the administration of acetazolamide (Diamox) which produces both of these effects although by different mechanisms. These attempts were exploratory in nature; they were carried out only for brief periods and without strict or adequate control of a number of factors, such as diet, fluid intake, etc., since it was desired to determine only if clear-cut and marked effects could be elicited.

Alterations in fluid intake did not appear to influence the levels of drug in the plasma or the rate of its excretion in the urine to any appreciable extent. Oral administration of alkalis for brief periods in amounts sufficient to alkalinize the urine had some effect in increasing the excretion of the drug. The combined effect of water diuresis and excretion of a moderately alkaline urine,

as accomplished here by the administration of a single dose of acetazolamide, did result in a temporary increase in the rate of excretion of sulfamethoxypyridazine. The increased excretion of drug that resulted following the single dose of acetazolamide, however, was only temporary and had only a minor over-all effect on the levels of the sulfonamide in plasma and on the total time during which the drug continued to be excreted in the urine. The effect of repeated doses was not investigated in the present study.

Should the freedom from untoward effects from small doses such as 1.0 Gm. every other day or 0.5 Gm. daily in adults be confirmed when they are used over long periods and in large numbers of patients, sulfamethoxypyridazine should prove useful in the continuous prophylaxis of streptococcal infections and hence of recurrences of rheumatic fever and perhaps also in the prevention or suppression of other subacute or chronic bacterial infections for which sulfonamides have proved useful. In single doses of 1.0 Gm. it may be an ideal agent for the prophylaxis or eradication of meningococcal infections and carriers from members of families or communities in which cases are occurring or carriers are demonstrated; this would be particularly applicable to schools, military installations, or similar assemblages. Trials of the use of this agent would therefore seem to be warranted in such situations, and perhaps also in the suppression or prevention of relapses of chronic urinary tract infections, particularly after the infections have already been actively treated and "cured" or suppressed with the same or other effective agents.

SUMMARY AND CONCLUSIONS

Absorption and urinary excretion of a new, long-acting antibacterial sulfonamide, sulfamethoxypyridazine (Kynex) was studied after a single oral dose of 3.0 Gm. in 6 normal young men and after repeated doses of 1.0 Gm. every 48 hours in 7 patients.

After a dose of 3.0 Gm., maximum levels of nearly 26 mg. per 100 ml. were demonstrated in the plasma within about 7 hours; these fell gradually to insignificant levels over a period of 8 days or longer. Nearly all of the administered drug was recovered in the urine, where detectable amounts were still demonstrable 10 to 14 days after the dose. Between 10 and 15 per cent of the drug in the plasma and more than one-half of that in the urine were found in the conjugated form.

Diuresis resulting from increased intake of fluid had no appreciable effect, and the oral administration of alkalis for brief periods had only a slight effect in increasing the rate of excretion of the drug. A definite increase in its clearance from the plasma accompanied the diuresis and alkalinization of the urine that followed the administration of 0.5 Gm. of acetazolamide (Diamox).

The administration of 1.0 Gm. every 48 hours in patients produced average plasma levels ranging between 5 and 12.5 mg. of total sulfamethoxypyridazine per 100 ml. and also significant concentrations in their urine in the intervals between doses.

The only untoward effect noted was a moderately severe headache in most of the subjects who received the dose of 3.0 Gm. No untoward effects were experienced by the patients who received repeated doses of 1.0 Gm. every other day.

Trials of sulfamethoxypyridazine in proper dosage seem to be warranted in order to determine its true value for brief or long-term prophylaxis or for treatment of sulfonamide-susceptible infections.

REFERENCES

1. Maren, T. H., and Mayer, E.: Personal communication, April 27, 1955.
2. Litchfield, J. T., Jr.: Properties of a New Antibacterial Sulfonamide, Kynex: 3-Sulfanilamido-6-Methoxy-Pyridazine. XX. International Physiological Congress, Brussels, Belgium, 1956.
3. Nichols, R. L., Jones, W. F., Jr., and Finland, M.: Sulfamethoxypyridazine: Preliminary Observations on Absorption and Excretion of a New, Long-Acting Antibacterial Sulfonamide, Proc. Soc. Exper. Biol. & Med. **92**: 637-640, 1956.
4. Bratton, A. C., and Marshall, E. K.: A New Coupling Component for Sulfanilamide Determination, J. Biol. Chem. **128**: 537-550, 1939.
5. Folin, O., and Wu, H.: A Simplified and Improved Method for Determination of Sugar, J. Biol. Chem. **41**: 367-374, 1920.
6. Peterson, O. L., Strauss, E., Taylor, F. H. L., and Finland, M.: Absorption, Excretion and Distribution of Sulfadiazine (2-Sulfanilamido Pyrimidine), Am. J. M. Sc. **201**: 357-367, 1941.

SERUM VITAMIN B_{12} LEVEL AND BINDING SUBSTANCE OF TUBERCULOUS PATIENTS WITH AND WITHOUT LIVER DISEASE

ROBERT L. DAVIS, SC.D., ROBERT C. DUVALL, M.D., AND BACON F. CHOW, PH.D.
BALTIMORE, MD.

INTRODUCTION

IN TWO previous communications,[1-2] data were presented to demonstrate that trace amounts of certain biologic compounds such as heparin had the capacity to prevent the uptake of radioactive vitamin B_{12} labeled with cobalt^{60} by the resting cells of *Lactobacillus leichmannii* ATCC 4797. In the absence of such compounds, these bacterial cells can remove unbound vitamin B_{12} rapidly and quantitatively from very dilute solutions. The uptake of vitamin B_{12} decreases proportionately as the amount of the binding substance incorporated in the solution is increased. In a third communication,[3] a method was described to quantitate the unsaturated vitamin B_{12} binding capacity of human serum which is one of the biologic systems in which vitamin B_{12} binding substances are present in varying amounts. In all the studies, the vitamin B_{12} binding levels are measurements of the unsaturated binding capacity of the serum samples. This is accomplished by adding Co^{60}-labeled vitamin B_{12} in excess of the serum binding capacity and measuring the free radioactive vitamin B_{12} by the microbial adsorption technique. The serum vitamin B_{12} binding level is the resultant of two variables: the amount of vitamin B_{12} already present, and the quantity of vitamin B_{12} binding substances. This procedure was employed to study the vitamin B_{12} binding levels of serum samples obtained from normal blood donors and from tuberculous, diabetic, and elderly human adults. It was observed that there were differences in the vitamin B_{12} binding levels among the various test groups. The tuberculous group has only a slightly higher mean level, but the range was of greater spread than those of the blood donor group. On further observation, it was noted that almost all serum samples which possessed very low binding levels also exhibited hyperbilirubinemia. Several months later, a small outbreak of hepatitis had developed and serum samples obtained from these cases showed a pattern consistent with the earlier findings. The results of serial assays of vitamin B_{12} binding levels, C-reactive protein, and liver function tests of 15 tuberculous patients with hepatitis, together with related studies, are reported in this communication. C-reactive protein serum determinations were included in this study to

From the Veterans Administration Hospital and The Johns Hopkins University School of Hygiene and Public Health, Department of Biochemistry, Baltimore, Md.

Submitted in part for presentation at the Symposium on the Clinical Aspects of Vitamin B_{12}, May 27, 1955, School of Hygiene, The Johns Hopkins University; and at the Maryland Section of the American Chemical Society, Nov. 18, 1955, Baltimore, Md.

Received for publication Aug. 1, 1956.

determine whether tuberculous patients with virus hepatitis possess this "acute phase protein" observed to be absent by Havens[4] in 90 patients in various phases of viral hepatitis. Other reports[5-6] did not confirm this finding.

MATERIALS AND METHODS

*Vitamin B_{12}.**—The vitamin B_{12} containing cobalt^{60} employed in this study had a specific activity of approximately 800 μc per milligram. The concentration of the diluted vitamin B_{12} solution employed in the vitamin B_{12} binding assays was estimated by the microbiologic assay with *Lactobacillus leichmannii* according to the method of Skeggs and co-workers.[7]

Resting Cells.—The preparation of "resting" cells of *Lactobacillus leichmannii* ATCC 4797 was described previously.[3-8] The "resting" cells once prepared can be kept at 4° C. for several days, after which time washing with distilled water or isotonic saline solution will restore the greater part of the vitamin B_{12} uptake capacity.

Serum.—Whole blood was obtained by venipuncture from fasting patients. Serum obtained by centrifugation was stored in a frozen state until assayed. It was observed previously that serum samples could be preserved in this manner for several years without appreciable loss of vitamin B_{12} binding capacity. Likewise, serum samples allowed to remain at room temperature for short periods of time (two hours) lost little, if any, of the binding capacity.

The procedure for the quantitative determination of the vitamin B_{12} binding capacity of serum has been described previously.[3] However, some minor modifications have been adopted. In order to utilize less serum and radioactive vitamin B_{12}, half quantities were employed in this study, namely, 0.5 ml. serum and 2.0 Mμg of vitamin B_{12}. With serum samples containing very low vitamin B_{12} binding capacities, greater quantities of serum may be used, and for those of high binding capacity 4 Mμg or more of radioactive vitamin B_{12} may be added to the serum.

Liver function tests performed were those commonly employed in clinical laboratories—BSP, cephalin flocculation, and thymol turbidity.

C-reactive protein: The precipitin test method as described by Muschel and Weatherwax[9] was employed in this study.

RESULTS

The comparison of the serum vitamin B_{12} binding levels obtained during the acute and convalescent stages of liver disease is presented in Fig. 1. The striped bars represent the initial binding during the height of bilirubinemia (acute stage); the dotted bars, the binding when liver function test values approached or were normal (convalescent stage). The last two bars concern a patient with cirrhosis whose vitamin B_{12} binding capacity was elevated throughout the entire investigation.

The data presented in Fig. 2 demonstrate the relationship between the serum vitamin B_{12} binding and the total serum bilirubin values of tuberculous patients with hepatitis. These curves, which plot serum bilirubin level and serum vitamin B_{12} binding capacity against time (weeks), reveal that as the serum bilirubin level decreased, the vitamin B_{12} binding capacity increased markedly. This consistent pattern was observed in more than fifteen cases of virus hepatitis. In Table I are listed the averages of the vitamin B_{12} binding and bilirubin levels during the acute and convalescent stages of hepatitis. During the acute stage of hepatitis, the average values of the bilirubin and

*Kindly supplied by Dr. Charles Rosenblum of Merck & Co., Rahway, N. J.

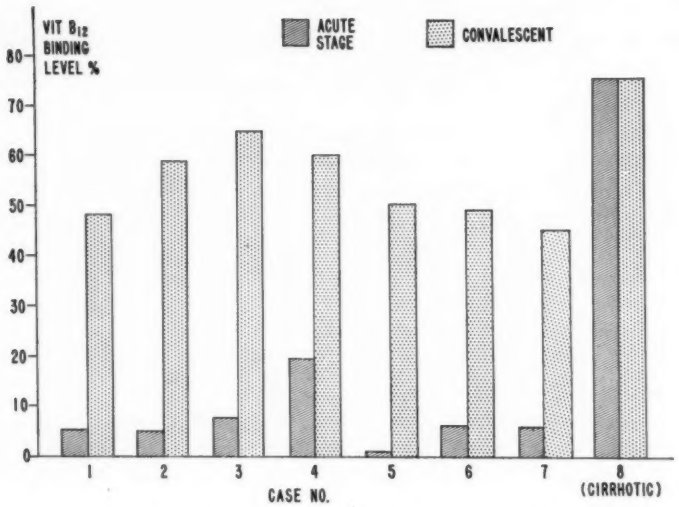


Fig. 1.—Vitamin B_{12} binding levels during and after hepatitis.

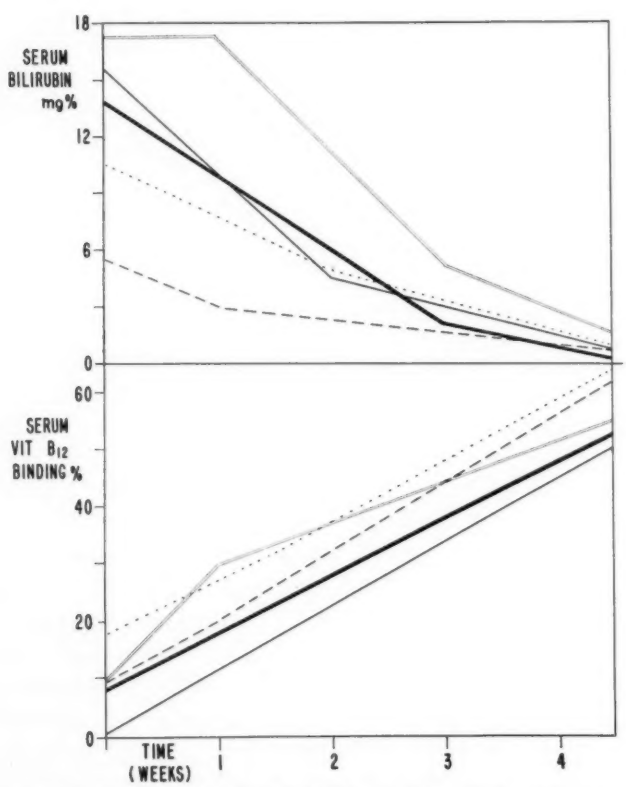


Fig. 2.—Serum bilirubin and vitamin B_{12} binding levels of tuberculous patients with hepatitis.

vitamin B_{12} binding levels were 9 mg. per cent and less than 10 per cent, respectively, whereas after several weeks the bilirubin values decreased to 1 mg. per cent and the vitamin B_{12} binding capacities increased over fivefold.

TABLE I. MEAN SERUM VITAMIN B_{12} BINDING AND BILIRUBIN VALUES

GROUP	NUMBER	SERUM BILIRUBIN MG. % MEAN	SERUM VITAMIN B_{12} BINDING CAPACITY % MEAN
Acute stage of hepatitis	15	9.0 ± 3.0*	10 ± 3
Convalescent stage of hepatitis	15	1.0 ± 0.3	55 ± 5
Blood donors	40	<1.0	45 ± 8

*Standard deviation.

TABLE II. SERUM VITAMIN B_{12} BINDING CAPACITY AND CONTENT DURING COURSE OF VIRAL HEPATITIS

PATIENT	SERUM VITAMIN B_{12} BINDING CAPACITY (%)		SERUM VITAMIN B_{12} LEVEL ($\mu\mu$G/ML.)	
	ACUTE STAGE	CONVALESCENT STAGE	ACUTE STAGE	CONVALESCENT STAGE
1	<10	45	600	200
2	0	50	>1,500	200
3	<10	50	600	215
4	<10	55	750	250

Table II presents data on the relationship between the serum vitamin B_{12} level and vitamin B_{12} binding capacity of several tuberculous patients with virus hepatitis. It can readily be observed that as the serum binding capacity of the patient increased from a very low value to a higher one, the vitamin B_{12} serum content fell. In all the serial assays performed on serum samples from cases of infectious hepatitis or homologous serum jaundice, inverse relationships between serum level and binding capacity of vitamin B_{12} were found. Table III demonstrates this further. In this table, the vitamin B_{12} binding, vitamin B_{12} level, C-reactive protein, and clinical diagnosis of several patients are compared. The data reported in this table clearly indicate the lack of correlation between C-reactive protein and vitamin B_{12} binding in the tuberculous patients with hepatitis and between C-reactive protein and vitamin B_{12}

TABLE III. COMPARISON OF SERUM VITAMIN B_{12} BINDING CAPACITY, LEVEL, C-REACTIVE PROTEIN OF TUBERCULOUS PATIENTS WITH AND WITHOUT LIVER DISEASE

PATIENT	DATE	SERUM VITAMIN B_{12} LEVEL ($\mu\mu$G/ML.)	SERUM VITAMIN B_{12} BINDING (%)	C-REACTIVE PROTEIN	DIAGNOSIS
1	7- 1	750	<10	+	H & TB
2	1-31	>600	<10	0	H & TB
2	2-28	200	45	0	H & TB
3	5- 6	>1,500	<10	+	H & TB
3	5- 9	>1,500	<10	+	H & TB
3	5-16	1,500	<10	++	H & TB
3	5-26	400	30	++	H & TB
3	7-26	200	50	++	H (convales.)
4	5-20	60	30	++	TB
5	6-21	80	45	0	TB
6	12-30	<50	95	++++	TB
7	5-17	76	18	+++	TB
8	10- 5	300	80	++	C & TB
9	7-27	300	70	+++	C & TB
10	9-21	190	80	++	C & TB

TB = Pulmonary tuberculosis. H = Viral Hepatitis. C = Cirrhosis.

level. Likewise, in the limited data presented in Table III, there was no direct relationship between the serum vitamin B_{12} binding and total vitamin B_{12} level except in hepatitis patients (patients 1, 4, and 6).

The data also indicate that cirrhotic serum samples exhibit a much greater variation in the B_{12} binding and B_{12} levels than do those of the infectious hepatitis and homologous serum jaundice samples. It may be that the serum samples employed for the study of these levels, in the case of cirrhotic patients, were not all obtained during the acute stage of the disease. Nevertheless, most of the studies were initiated on cirrhotic serum samples obtained when the liver function tests were at the height of abnormal values.

Changes in vitamin B_{12} binding capacity were compared with several of the commonly employed liver function tests. Initially in all patients investigated, prior to the administration of blood transfusions, the vitamin B_{12} binding capacity was elevated or normal. The vitamin B_{12} binding markedly decreased with the onset of homologous serum jaundice. As the hepatocellular disease subsided, and the patient improved clinically, the serum B_{12} binding capacity slowly returned to a normal or high range and remained stabilized. During the course of convalescence, several mild relapses were observed. Such relapses resulted in decreased vitamin B_{12} binding levels.

In one case of leptospirosis in which abnormal liver function tests such as elevated bilirubin, bromsulfalein, and thymol turbidity were observed, the serum vitamin B_{12} binding capacity was elevated. This was in marked contrast to the decreased binding capacity observed in hepatitis. It has been observed by electrophoretic study that sera from patients with Weil's disease show an increase in alpha-2 globulin. This fraction has been demonstrated to bind vitamin B_{12} by several investigators.[10-12, 15, 16]

DISCUSSION

An altered capacity of serum to bind vitamin B_{12} has been demonstrated in certain disease states.[10-13] In an earlier study,[3] it was observed that the capacity of tuberculous serum samples to bind vitamin B_{12} varied to a marked degree. The vitamin B_{12} binding levels of human serum samples of tuberculous patients with liver disease may vary considerably, a fact which is similar in several respects to the results of Greenspan's[14] investigation of serum mucoprotein levels in liver disease.

The change in the vitamin B_{12} binding capacity of patients with certain diseases may reflect the clinical status. The net vitamin B_{12} binding capacity may be the result of several distinct phenomena. The reduction of binding capacity may be due to the decrease of serum γ 1 and 2 globulin fractions and of available binding sites by the increased serum vitamin B_{12} content resulting from the damage of liver cells. In some instances, this lowering of vitamin B_{12} binding capacity may be partially offset by an increase in binding substances due to some complicating disease present. Conversely, high binding capacity of serum samples may be due to decrease in total B_{12} content or increase in binding substance. The question still remains as to which of these

factors contributes the greatest influence on the resultant binding level. The elevation in the serum vitamin B_{12} level appears to be one of the main factors in virus hepatitis.

Differentation between hepatitis and cirrhosis, although not absolute, may be aided by observing the serum vitamin B_{12} binding capacities in hyperbilirubinemic patients. It was found that during the acute stage of hepatitis, at which time the bilirubin level was at its height, the serum vitamin B_{12} binding capacity was almost zero. On the other hand, in the limited number of patients with cirrhosis investigated, the serum vitamin B_{12} binding capacity as well as total vitamin B_{12} content did not always parallel the serum bilirubin concentrations. Lear and co-workers[12] noted that in 29 cases of hepatic cirrhosis there was a very wide range of serum vitamin B_{12} levels, and this observation has been our experience, too. In our cirrhotic patients the capacity to bind vitamin B_{12} did not drop as markedly (in some cases not at all) in spite of abnormal liver function tests. In general, the vitamin B_{12} levels were considerably lower and of greater spread than those of hepatitis group. More studies are in progress.

Havens reported that C-reactive protein which appears in the blood in response to the presence of an inflammatory reaction is not found in hepatitis. In more recent reports, several investigators employing more sensitive methods have found C-reactive protein in the serum samples of cases of hepatitis. Perlman and co-workers[17] reported in 1943 that C-reactive protein follows the alpha-globulin group in moving boundary electrophoresis. Per Hedlund and Inger Brattsen[18] by the employment of continuous zone electrophoresis reported in 1955 that this acute phase protein contained protein that migrated with the mobility of a gamma-globulin exclusively. Schaffner demonstrated by paper electrophoresis that the serum alpha globulin level dropped markedly during the acute state of hepatitis and rose to normal during convalescence. Pitney, Beard, and Sanneman[15] have shown by microbiologic and radioactive tracer techniques that the vitamin B_{12} activity of serum is distributed through alpha-1 and alpha-2 globulin fractions. Since our studies have indicated that during the acute stages of infectious hepatitis and homologous serum jaundice serum vitamin B_{12} binding capacities are very low, and that during the course of several inflammatory diseases they are elevated, it was of interest to determine if any direct relationship existed between vitamin B_{12} binding capacity and C-reactive protein. Furthermore, it would offer some indirect evidence as to whether C-reactive protein follows the alpha- or gamma-globulin groups. Gamma globulin has been shown by several workers to be a relatively poor vitamin B_{12} binder. If C-reactive protein should belong to and behave similarly to this fraction, the possibility of a direct relationship to vitamin B_{12} binding should be small. Preliminary experiments performed on 40 tuberculous patients with and without liver disease have indicated that no direct correlation exists between C-reactive protein and vitamin B_{12} binding. Also, tuberculous patients with homologous serum jaundice may or may not have C-reactive protein.

SUMMARY

1. During the height of hyperbilirubinemia, the serum vitamin B_{12} binding capacities of cases of tuberculosis with infectious hepatitis or homologous serum jaundice were very low, but they rose to higher levels during the course of convalescence.

2. The vitamin B_{12} levels were high during the acute stage and normal during convalescence.

3. A small number of cirrhotics presented no consistent pattern.

4. No correlation was observed between C-reactive protein and vitamin B_{12} binding capacity or level.

5. Several tuberculous patients with hepatitis were positive for C-reactive protein.

We appreciate the grant-in-aid from the Atomic Energy Commission.

REFERENCES

1. Chow, Bacon F., and Davis, Robert L.: Some Observations of Substances Combining With Vitamin B_{12}, Proc. of the 7th Annual Meeting of the National Vitamin Foundation, 1952, p. 17.
2. Davis, Robert L., and Chow, Bacon F.: Compounds Inhibiting the Uptake of Vitamin B_{12} by Resting Bacterial Cells, Fed. Proc. **12**: 1449, 1953.
3. Davis, Robert L., and Chow, Bacon F.: Vitamin B_{12} Binding Substances in Human Serum, Fed. Proc. **13**: 33, 1954.
4. Havens, W. P., Jr., Eichman, H. L., and Knowenton, M.: Failure to Find C-reactive Protein in Viral Hepatitis, Proc. Soc. Exper. Biol. & Med. **75**: 108-110, 1950.
5. Hedlund, P.: Appearance of Acute Phase Protein in Various Diseases, Acta Med. scandinav. Supplement 196-7, 579, 1947.
6. Berkowitz, Donald: Unpublished data.
7. Skeggs, H. R., Nepple, H. M., Valentik, K. O., Huff, J. W., and Wright, L. D.: Observation on the Use of Lactobacillus Leichmannii 4797 in the Microbiological Assay of Vitamin B_{12}, J. Biol. Chem. **184**: 211-224, 1950.
8. Davis, Robert L., and Chow, Bacon F.: Determination of Vitamin B_{12} by Means of Its Combination With Lactobacillus Leichmannii Resting Cells, Fed. Proc. **11**: 466, 1952.
9. Muschel, Louis H., and Weatherwax, Robert J.: Complement Fixation in the C-reactive Protein System, Proc. Soc. Exper. Biol. & Med. **87**: 191-193, 1954.
10. Beard, Marion F., Pitney, W. R., and Sanneman, E. H.: Serum Concentration of Vitamin B_{12} in Patients Suffering From Leukemia, Blood **9**: 789, 1954.
11. Pitney, W. R., and Beard, M. F.: Serum Vitamin B_{12} Concentrations in Pernicious Anemia, J. Lab. & Clin. Med. **42**: 928-929, 1953.
12. Lear, Arnold A., Harris, John W., Castle, William B., and Fleming, Eleanor M.: The Serum Vitamin B_{12} Concentration in Pernicious Anemia, J. Lab. & Clin. Med. **44**: 715-722, 1954.
13. Davis, Robert L., Duvall, Robert C., and Chow, Bacon F.: Study of Vitamin B_{12} Binding Levels in Tuberculous Patients With Hepatitis. Symposium on the Clinical Aspects of Vitamin B_{12}. May (1955). The Johns Hopkins University, Baltimore, Md.
14. Greenspan, E. M., Tepper, B., Terry, L. L., and Schoenbach, E. B.: The Serum Microproteins as an Aid in the Differentiation of Neoplastic From Primary Parenchymatous Liver Disease, J. Lab. & Clin. Med. **39**: 44-56, 1952.
15. Pitney, R., Beard, M., and Sanneman, E.: In Vitro Binding Capacity of Human Serum for Vitamin B_{12}, J. Clin. Invest. **32**: 595, 1953.
16. Pitney, W. R., Beard, M. F., and Van Loon, E. J.: Observations on the Bound Form of Vitamin B_{12} in Human Serum, J. Biol. Chem. **207**: 143-152, 1954.
17. Perlman, E., Bullowa, J. G., and Goodkind, R.: Immunological and Electrophoretic Comparison of the Antibody to C Polysaccaride and the C-reactive Protein of Acute Phase Serum, J. Exper. Med. **77**: 97-110, 1943.
18. Hedlund, P., and Brattsen, Inger: Electrophoretic Analysis of Human Acute Phase Protein, Scandinav. J. Clin. & Lab. Invest. **1**: 99-100, 1955.

THE EFFECTS OF SODIUM LACTATE ON RENAL METABOLISM IN PATIENTS WITH KIDNEY DISEASE

ARCHER P. CROSLEY, JR., M.D., JOHN F. BROWN, M.D.,* BENJAMIN SCHUSTER, M.D.,* DEAN A. EMANUEL, M.D., HERMAN TUCHMAN, M.D.,* CESAR CASTILLO, M.D., AND GEORGE G. ROWE, M.D.
MADISON, WIS.

SODIUM lactate is utilized in the treatment of abnormal metabolic disease states, cardiac arrhythmias, and renal disease. Despite its widespread usage, studies of its effects on renal metabolism, renal hemodynamics, and the maximal secretory capacity of sodium para-aminohippurate (Tm_{PAH}) have been largely confined to in vitro and in vivo experiments in animal preparations.[1, 2] Only one paper has reported the alterations produced by this agent on Tm_{PAH} in patients with renal disease.[3]

The purpose of this paper is twofold: (1) To report the effects of intravenous sodium lactate on renal hemodynamics, renal metabolism and Tm_{PAH} in patients with renal disease. (2) To demonstrate the use of the nitrous oxide technique for the determination of renal blood flow,[4] as adapted to human use,[5] as an adjunct to studies of renal metabolism in man.

METHODS

The study was performed on six fasting patients from the medical wards of the University Hospitals. All individuals had renal disease. The exact diagnoses are given in Table I.

Previously described techniques[5-7] and right renal vein catheterization were utilized to determine glomerular filtration rate (GFR), total renal blood flow (RBF_{PAH}) ($Fick_{PAH}$), renal blood flow per 100 grams of kidney (RBF_{N_2O}), renal arteriovenous oxygen differences ($A\text{-}R_{O_2}$), renal oxygen consumption (Q_{O_2}), and the tubular maximal secretory capacity for PAH(Tm_{PAH}).

Following three 15-minute control periods during which conventional methods for the study of renal hemodynamics, metabolism, and Tm_{PAH} were used, a nitrous oxide renal blood flow was performed. At the completion of the latter M/6 sodium lactate was infused intravenously at a rate of approximately 5 ml. per minute for a period of one hour. During this interval, 3 additional 15-minute periods were carried out according to conventional techniques, followed by a second nitrous oxide renal blood flow determination.

From The Cardiovascular Laboratory and Department of Medicine, University of Wisconsin School of Medicine, Madison.

This investigation was supported in part by grants from the Wisconsin Heart Assn., Wisconsin Alumni Research Foundation, and The National Heart Institute (H-1495C), United States Public Health Service.

Received for publication Aug. 10, 1956.

*Heart Trainee of National Heart Institute, U. S. Public Health Service, 1955.

TABLE I. EFFECTS OF SODIUM LACTATE ON RENAL FUNCTION AND METABOLISM IN KIDNEY DISEASE

PATIENT	PERIOD	GFR ML./MIN.	GFR % Δ	RBF_{PAH} ML./MIN.	RBF_{PAH} % Δ	RBF_{N_2O} ML./100 G./MIN.	RBF_{N_2O} % Δ	A-Ro_2 VOL. %	A-Ro_2 % Δ	$Qo_{2\ PAH}$ ML./MIN.	$Qo_{2\ PAH}$ % Δ	$Qo_{2\ N_2O}$ ML./100 G./MIN.	$Qo_{2\ N_2O}$ % Δ	Tm_{PAH} MG./MIN.	Tm_{PAH} % Δ	$\frac{Qo_{2\ PAH}}{Tm}$ ML./MG.	$\frac{Qo_{2\ PAH}}{Tm}$ Δ %
1. 29-W-M	C	95		1,215		398		1.2		14.6		4.8		85		0.172	
Benign nephrosclerosis	L	83	−14	1,075	−12	394	− 1	1.5	+25	16.1	+10	5.9	+21	87	+ 2	0.185	+ 8
2. 44-W-M	C	60		630		336		2.1		13.2		7.1		69		0.192	
Benign nephrosclerosis	L	72	+20	680	+ 8	348	+ 3	2.2	+ 5	15.0	+14	7.7	+ 8	81	+17	0.185	− 4
3. 36-W-M	C	79		1,935		398		1.3		25.2		5.2		94		0.266	
Diabetic nephropathy	L	80	+ 2	2,050	+ 6	406	+ 2	1.5	+15	30.8	+22	6.1	+17	116	+24	0.268	0
4. 31-W-M	C	43		740		266		1.0		7.4		2.7		53		0.140	
Chronic glomerulonephritis	L	47	+ 9	705	− 5	225	−15	1.5	+50	10.6	+14	3.4	+26	60	+13	0.177	+27
5. 35-W-M	C	70		---		378		1.2		---		4.5		100		---	
Chronic glomerulonephritis	L	85	+21	---	---	360	− 5	1.3	+ 8	---	---	4.7	+ 4	111	+11	---	---
6. 43-W-M	C	107		1,390		---*		1.0		13.9		---		86		0.162	
Benign nephrosclerosis	L	116	+ 8	1,155	−24	---	---	1.5	+50	17.3	+24	---	---	95	+11	0.178	+10
Δ % $\bar{x}$			+ 8		− 5		− 3		+26		+17		+15		+17		+ 8
p			>0.3		>0.4		>0.4		<0.05		<0.01		<0.02		<0.01		>0.3

C = Average of control study.
L = Average of lactate study.
GFR = Glomerular filtration rate Inulin clearance.
RBF_{PAH} = Total renal blood flow

$$\frac{UV_{PAH}}{\frac{A_{PAH} - R_{PAH}}{1 - Hct.}}$$

RBF_{N_2O} = Renal blood flow (nitrous oxide)
A-Ro_2 = Renal arteriovenous oxygen difference.
$Qo_{2\ PAH} = RBF_{PAH} \times A\text{-}Ro_2$
$Qo_{2\ N_2O} = RBF_{N_2O} \times A\text{-}Ro_2$
Tm_{PAH} = Tubular maximal secretory capacity of PAH.
% Δ = Per cent change from mean control value.
*Loss due to laboratory error.
Δ % $\bar{x}$ = Mean change of group from individual mean control values.
p = Level of statistical significance.

RESULTS

The results are summarized in Table I.

Following the infusion of M/6 sodium lactate, Tm_{PAH} increased significantly ($\Delta\%$* = +17, p = < 0.01). This rise was not associated with significant changes in the renal blood flow as determined by the Fick PAH method ($\Delta\%$ = –5, p = > 0.4) or by the nitrous oxide method ($\Delta\%$ = –3, p = > 0.4). However, the renal arteriovenous oxygen difference ($A\text{-}R_{O_2}$) increased significantly ($\Delta\%$ = +26, p = < 0.05) so that the product of the renal blood flow by either technique and the $A\text{-}R_{O_2}$, namely, renal oxygen consumption (Q_{O_2}), increased significantly ($Q_{O_{2\ (PAH)}}$ $\Delta\%$ = +17, p = < 0.01; $Q_{O_{2\ (N_2O)}}$ $\Delta\%$ = +15%, p = < 0.01). The increase in renal oxygen consumption paralleled the rise in Tm_{PAH} so that no significant change occurred in the ratio Q_{O_2}/Tm ($\Delta\%$ = +8%, p = > 0.3).

DISCUSSION

The results show that the mean percentage change in renal blood flow by the N_2O technique was not significantly different from that obtained by the Fick PAH method (d = 2%, p = > 0.1). Therefore since the same figure for $A\text{-}R_{O_2}$ was used in the general equation: $RBF \times A\text{-}R_{O_2} = Q_{O_2}$, the mean percentage changes in the renal oxygen consumption, regardless of the manner in which the blood flow was determined, were not significantly different (d = 2, p = > 0.1). Since the conclusions, regardless of the technique utilized for renal blood flow, are the same, namely, that renal blood flow remained unchanged while renal oxygen consumption increased significantly, it appears that there is support for the validation of the N_2O renal flow method in studies of renal metabolism in man.

The observed increase in renal oxygen consumption might be related to an increase in renal work associated with an enhanced secretory transport of PAH, to increased renal work associated with the external excretion of the sodium lactate or its metabolites, or to the metabolism of lactate by the kidney. The first possibility is probably negated by the previous work of Clark and Barker,[8] who demonstrated no change in renal Q_{O_2}, as the secretory mechanism for PAH was saturated by increasing plasma levels of this substance. Furthermore this would be compatible with calculations of the present data in which the correlation coefficient between the changes in $Q_{O_{2\ (PAH)}}$ and the changes in Tm_{PAH} was only 0.55 (p = > 0.1). The idea that the increased Q_{O_2} might be related to an increased external excretion of sodium lactate or its metabolites is not borne out by previous studies which indicate no correlation between an enhanced excretion of anions and cations and renal oxygen consumption.[9, 10] The most plausible explanation would appear to be that the increased Q_{O_2} was related to the metabolism of this agent by the kidney.

The increases in renal oxygen consumption and Tm_{PAH} following intravenous sodium lactate in these patients are in accord with the work of previous

*$\Delta\%$ = Mean percentage change of the group from individual mean control values.

investigations in tissue slices[1] and in vivo studies in the dog.[2] Thus it would appear that the diseased kidney is not abnormal in its response to this agent so far as these functions are concerned.

SUMMARY AND CONCLUSIONS

The intravenous administration of M/6 sodium lactate to 6 patients with renal disease resulted in an enhanced renal oxygen consumption. The latter increase was probably related to the metabolism of sodium lactate by the kidney and was reflected by an increase in renal arteriovenous oxygen difference, since no significant changes occurred in renal blood flow. This response in Q_{O_2}, as well as the increase in Tm_{PAH}, demonstrates that the diseased kidney is not abnormal in its response to this agent. Since the results and conclusions were the same whether the nitrous oxide or Fick PAH methods were used for the determination of renal blood flow, the data support the use of the former technique as a new means for studies of renal oxygen consumption in man.

We wish to thank Mrs. Phyllis Fosshage, Mrs. Audrey Peterson, Mrs. R. Gilis, Miss T. Wuebben, Miss V. Putna, and Mrs. M. Dahl for their technical assistance in this work.

REFERENCES

1. Cross, R. J., and Taggart, J. V.: Renal Tubular Transport: Accumulation of P-Aminohippurate by Rabbit Kidney Slices, Am. J. Physiol. **161:** 181-190, 1950.
2. Mudge, G. H., and Taggart, J. V.: Effect of Acetate on the Renal Excretion of P-Aminohippurate in the Dog, Am. J. Physiol. **161:** 191-197, 1950.
3. Clark, J. K., Barker, E. S., and Crosley, A. P., Jr.: Acute Responses of Tm_{PAH} and Glomerular Filtration Rate to Intravenous Lactate in Uremic Patients, J. Clin. Invest. (abstract), 1951.
4. Conn, H. L., Jr., Anderson, W., and Arena, S.: Gas Diffusion Technique for Measurement of Renal Blood Flow With Special Reference to the Intact Anuric Subject, J. Appl. Physiol. **5:** 683, 1953.
5. Crosley, A. P., Jr., Brown, J. F., Huston, J. H., Emanuel, D. A., Tuchman, H., Castillo, C., and Rowe, G. G.: The Adaptation of the Nitrous Oxide Method to the Determination of Renal Blood Flow and In Vivo Renal Weight in Man. J. Clin. Invest. **35:** 1340, 1956.
6. Goldring, W., and Chasis, H.: Hypertension and Hypertensive Disease, New York, 1944, The Commonwealth Fund.
7. Crosley, A. P., Jr., Rowe, G. G., and Crumpton, C. W.: The Metabolic and Hemodynamic Response of the Human Hypertensive Kidney to a Standard Intravenous Dose of 1-Hydrazinophthalazine (Hydralazine), J. Lab. & Clin. Med. **44:** 104, 1954.
8. Clark, J. K., and Barker, H. G.: Studies of Renal Oxygen Consumption in Man. I. The Effect of Tubular Loading (PAH), Water Diuresis and Osmotic (Mannitol) Diuresis, J. Clin. Invest. **30:** 745-750, 1951.
9. Crosley, A. P., Jr., and Rowe, G. G.: Hemodynamic and Metabolic Response of Human Hypertensive Kidney to Sodium p-Sulfamyl Benzoate, Proc. Soc. Exper. Biol. & Med. **89:** 26-28, 1955.
10. Barker, E. S., Clark, J. K., and Barker, H. G.: The Effects of Probenecid on Renal Hemodynamics and Metabolism, Fed. Proc. **12:** 9, 1953.

STUDIES ON ANTI-V: A RECENTLY DISCOVERED Rh ANTIBODY

ELOISE R. GIBLETT, M.D., JEANNE CHASE, B.S., AND ARNO G. MOTULSKY, M.D.
SEATTLE, WASH.

A NEW blood group antibody, "anti-V," was recently described by DeNatale and associates[1] in the serum of a multiple transfused, Rh negative, white man. This antibody delineated the new antigen "V," occurring in about one-third of Negroes but rarely in white people. Family studies revealed the V gene to be inherited as a Mendelian dominant related to the Rh system. When the V gene was present, it was invariably associated with c and e on the same chromosome.

By the employment of a panel of group O cells of known genotype,* two further examples of anti-V were encountered in this laboratory within a period of six months.† Using the serum from one of these patients, we were able to carry out investigations concerning the racial distribution of the V antigen and its relation to the Rh blood group system. Other studies to determine the incidence, serologic nature, and destructive capacity of anti-V in vivo were also performed. These investigations form the basis for this paper.

CASE REPORTS

I. H., an elderly white man, was admitted to a local hospital in May, 1955, because of severe hematuria and anemia. His pertinent history included a leg amputation ten years previously with subsequent complications requiring the transfusion of 22 units of blood, several of which were Rh positive. During his 1955 hospitalization he received eight units of blood, but he pursued a very rapid course and expired without an adequate diagnosis. It was because of difficulties encountered in cross-matching that his blood was investigated. His red cells were group A; the Rh genotype was R′r (Cde/cde). His serum contained both anti-D and anti-V.

J. B., a young white man with congenital nonspherocytic hemolytic anemia, was found on routine serologic study to have anti-D and anti-V in his serum. His sensitization had occurred twelve years previously, when he received ten units of blood following a war injury. His red cells were group A, Rh negative (rr), kk, Fy^a positive, V negative. This patient had mild icterus and reticulocytosis as well as moderate splenomegaly. Despite evidence of active red cell destruction, his hematocrit remained between 40 and 45. Blood drawn from this patient was the source of anti-V used in subsequent studies, and he was the subject for red cell survival experiments.

LABORATORY INVESTIGATIONS

Methods.—

1. *Serologic studies:* Preliminary experiments indicated that the anti-V in J. B.'s serum was not demonstrable as a saline agglutinin, and that it reacted very weakly with

From the King County Central Blood Bank and the Department of Medicine, University of Washington School of Medicine, Seattle, Wash.
Received for publication Aug. 11, 1956.
*"Panocell" from the Knickerbocker Foundation.
†Kindly confirmed by Dr. Amos Cahan.

albumen-suspended V positive cells. However, when the antiglobulin technique was used, the cells were strongly agglutinated. The antibody had a titer of 1:8 with A, Rh negative, V positive cells both before and after absorption with B, Rh positive cells. This absorbed serum was used on all subsequent testing. Anti-V titrations were carried out simultaneously on serum specimens kept frozen until used. The same A, Rh negative, V-positive cells were used in each titration.

To determine the type of antiglobulin reaction, serial dilutions of gamma globulin* (0.2 to 0.00002 per cent) were mixed with an equal volume of antiglobulin serum. This partially neutralized Coombs serum was then used to perform antiglobulin tests on V positive cells previously sensitized with anti-V, with use of appropriate controls.

It has been shown[2-4] that some incomplete antibodies require complement in order to sensitize red cells to antiglobulin serum. To test this requirement by anti-V, we inactivated the serum at 56° C. for 20 minutes. This serum was used to perform the antiglobulin test with V positive cells.

2. *Racial studies:* In order that the racial incidence of the V antigen might be determined, donors at the King County Central Blood Bank were screened with respect to color of skin, physical characteristics, and name into one of four groups: Caucasian, Negro, oriental, and American Indian. The large majority of orientals studied were Japanese or Filipino in origin. The Indian group included heterogeneous mixtures from many tribes in western America and Alaska. It was recognized that such rough classification would result in inaccuracies. However, since the primary interest was in finding V-positive subjects, appropriate inquiries could usually be made when necessary.

The Negro group was additionally studied by performing Rh genotyping on their cells with use of anti-C, D, E, c and e sera. It was soon found that the presence of incomplete anti-D in commercially prepared saline anti-C and anti-E would occasionally cause agglutination of saline-suspended R_oR_o (cDe/cDe) cells, as pointed out by Drs. R. R. Race and A. Cahan (personal communication). For this reason, all positive reactions with these sera were checked with at least one specimen of "slide test" pure anti-C or anti-E. Cells not agglutinated by saline anti-D were tested for the presence of D^u with the antiglobulin technique.

3. *Anti-V Incidence Studies:* During a period of six months, the serum from all blood bank donors with a history of transfusion or pregnancy, as well as the serum from all transfusion recipients, was screened with a pool of O cells of known genotype (including V) with use of the antiglobulin technique. Over 10,000 sera were tested in this manner.

4. *Red Cell Survival Studies:* The ability of anti-V to destroy V positive cells in vivo was tested in the following manner. Two milliliters of A, Rh negative, V positive cells, which had been tagged with 150 μc of high specific activity Cr^{51}, was mixed with 3 ml. of J. B.'s own cells, tagged with 15 μc P^{32} according to the method of Mollison and Cutbush.[3] Patient J. B. was placed in a prone position with in vivo scintillation counters directed over his spleen and liver. The tagged cells were injected intravenously, and the splenic and hepatic radioactivity was recorded for a period of one hour. Blood specimens were obtained at appropriate intervals so that the survival of both types of cells could be measured by counting in a well-type scintillation counter and Geiger counter, respectively.

Three months later, the patient was injected with his own cells tagged with Cr^{51}, so that the resultant splenic and hepatic radioactivity could be measured in the same manner. This provided a comparison of the fate of compatible versus incompatible cells.

RESULTS

1. *Serologic Properties of Anti-V.*—Using the antiglobulin technique, we found that J. B.'s serum had an initial anti-V titer of 1:8. Nine days after injection of 2 ml. of V-positive cells, the titer was 1:16. Three months later, it was 1:32. The addition of gamma globulin to antiglobulin serum completely destroyed its ability to agglutinate V-positive cells sensitized with anti-V.

*Kindly supplied by the Cutter Laboratories.

Heat inactivation of the anti-V serum had no effect on its ability to coat susceptible cells. These data provide good evidence that the J. B. anti-V is a gamma globulin which does not require complement.

2. *Racial Group Distribution of the V Gene.*—Table I shows the incidence of the V phenotype in the four described racial groups. Of the two American Indians, one Caucasian and one oriental (a sailor in the Philippine navy) who were V positive, there was no history of Negro ancestry. Three of these four individuals had the Rh genotype R_0r (cDe/cde)* or R_1r (or CDe/cde).† The fourth V-positive subject (of mixed Indian and Spanish ancestry) was originally thought to have the genotype R_1R_2 (CDe/cDE). However, Dr. Ruth Sanger absorbed anti-f serum with these cells, and she concluded that the probable genotype was R_zr (CDE/cde).‡

TABLE I. INCIDENCE OF THE ANTIGEN V IN RED CELLS OF VARIOUS RACIAL GROUPS

RACIAL GROUP	V POSITIVE	V NEGATIVE
Orientals	1	272
American Indians	2	174
Caucasians	1	514
Negroes	94	233

TABLE II. V GENE INCIDENCE IN THREE GROUPS OF NEGROES

	WEST AFRICA*	NEW YORK*	SEATTLE
Sample size	150	168	327
V Positive	0.4000	0.2679	0.2875
V Negative	0.6000	0.7321	0.7125
V Gene frequency†	0.2254	0.1444	0.1559

*From DeNatale, Cahan, Jack, Race, and Sanger, J. A. M. A. **159**: 247, 1955.
†V gene frequency = 1 - $\sqrt{}$ V negative phenotype frequency.

The high incidence of V-positive Negroes is confirmed in Table I. Table II is a comparison of the previously reported V gene frequency of Negroes from West Africa and New York[1] and the V gene frequency found in the Seattle group. It can be seen that there is very good correlation between the two American groups.

TABLE III. Rh GENOTYPE FREQUENCY AND THE DISTRIBUTION OF THE V PHENOTYPE AMONG THREE GROUPS OF NEGROES

	WEST AFRICA†				NEW YORK†				SEATTLE			
GENOTYPE*	NO.	%	NO. V POS.	% V POS.	NO.	%	NO. V POS.	% V POS.	NO.	%	NO. V POS.	% V POS.
cde/cde	8	5.3	2	25.0	10	6.0	3	30.0	27	8.2	4	14.8
cDe/cDe‡	86	57.3	41	47.6	81	48.2	23	28.4	144	44.0	62	43.1
CDe/cDe	27	18.0	7	25.8	41	24.4	12	29.2	83	25.4	18	21.6
cDE/cDe	21	14.0	9	42.8	20	11.9	7	35.0	39	11.9	10	25.6
Cde/cde	1	0.7	1	100.0	1	0.6	0	0	3	0.9	0	0
CDe/cDE	3	2.0	0	0	5	3.0	0	0	13	4.0	0	0
cDE/cDE	2	1.3	0	0	3	1.8	0	0	9	2.8	0	0
CDe/CDe	2	1.3	0	0	7	4.1	0	0	9	2.8	0	0
Totals	150		60		168		45		327		94	

*The most probable genotype (for Negroes) is given. Lack of the antibody anti-d precludes the possibility of distinguishing homozygotes (DD) from heterozygotes (Dd).
†From DeNatale and associates.[1]
‡Many of these Negroes would actually be cDe/cde. See text.

*Not serologically distinguishable from R_0R_0 (cDe/cDe).
†Not serologically distinguishable from R_1R_0 (CDe/cDe).
‡Not serologically distinguishable from R_zR_0 (CDE/cDe).

In Table III a comparison is made of the incidence of the Rh genotypes as well as the V phenotype for each Rh genotype, in the three Negro samples. There is good agreement between the results obtained in the New York and Seattle Negroes with respect to Rh genotype distribution. Furthermore, out of the total of 53 reported specimens not containing the gene combination ce on the same chromosome, there were no V positives. This gives excellent support to the hypothesis that the V gene never, or very rarely, occurs on a chromosome lacking c and e.

There is, however, a rather striking discrepancy between the New York and Seattle Negroes with respect to the distribution of the V gene among the various Rh genotypes. As pointed out by DeNatale and associates,[1] because V only occurs with the genes c and e, one would expect to find a higher frequency of the antigen V in rr (cde/cde), R_0R_0 (cDe/cDe), and R_0r (cDe/cde) cells than in R_1R_0 (CDe/cDe), R_1r (CDe/cde), R_2R_0 (cDE/cDe), and R_2r (cDE/cde) cells. They found the results in the New York Negroes (but not the West Africans) discrepant in this regard, and they suggested that further tests with New York Negroes would be helpful. Analysis of the data from Seattle Negroes showed that out of 171 with two chromosomes containing c and e, 66 (38 per cent) were V positive. However, out of 125 who had only one such chromosome, 28 (22 per cent) were V positive. When the results from both American groups are combined, the percentages are 35 per cent (V-positive Negroes with two eligible chromosomes) and 25 per cent (V-positive Negroes with one eligible chromosome). Thus a larger sample results in a better fit.

Analysis of the data in Table III shows that in the combined groups, there were 45 Negroes tested whose genotype was rr (cde/cde), and 311 who were either R_0R_0 (cDe/cDe) or R_0r (cDe/cde). From these figures, it is possible to arrive at an approximation of the percentage of each type of chromosome which actually carries the V gene. This will be more accurate for cde than for cDe, since the exact occurrence of the latter cannot be determined without using anti-d serum. However, Table IV indicates that even if the assumption is made that none of the R_0R_0 (cDe/cDe) specimens were actually R_0r (cDe/cde), the occurrence of V with cde is considerably less frequent than with cDe. When one considers that the pairing of the cde chromosome with about one-third of the cDe chromosomes* would give a falsely low value for

TABLE IV. APPROXIMATE OCCURRENCE OF THE CHROMOSOMES cdeV AND cDeV IN WEST AFRICAN,* NEW YORK,* AND SEATTLE NEGROES

	GENOTYPE cde/cde	GENOTYPE cDe/cDe (OR cDe/cde)
Sample size	45	311
V Positive	0.2000	0.4051
V Negative	0.8000	0.5949
V Gene frequency	0.1056	0.2287

*From DeNatale and associates[1]

*The frequency of cDe in West Africans is higher than in American Negroes, and cde is less frequent. Thus the number of cDe chromosomes (as well as CDe and cDE) paired with cde would be smaller in the African group than in the Americans.

the V gene frequency with cDe, it is obvious that even more cDe chromosomes than the calculated 22.8 per cent carry the V gene. These gene frequencies cannot be taken as exact, but the evidence is very good that V more often accompanies cDe than cde.

3. *Incidence of Anti-V.*—Screening the serum of more than 10,000 people (to be reported elsewhere) revealed a large number of antibodies, including the fairly unusual anti-fy^a, anti-Jk^a, and anti-Le^b. No further examples of anti-V were found despite the moderately large proportion of Negro donors in this area.

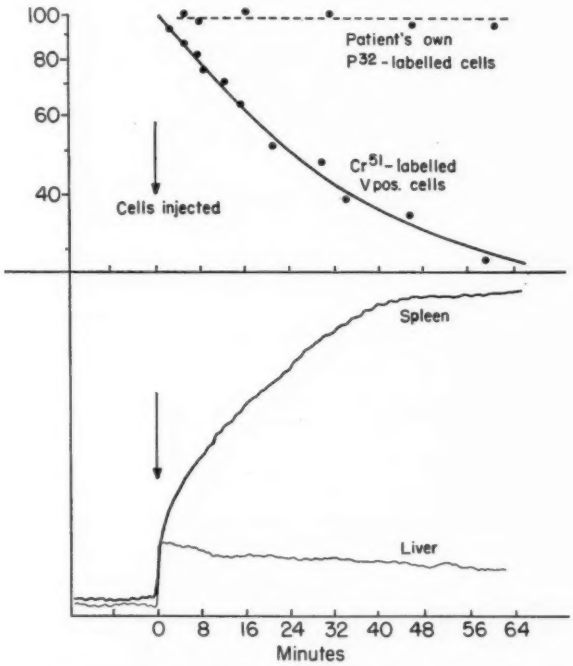


Fig. 1.—Above: Percentage of injected radioactivity in peripheral blood (log scale). Below: Radioactive uptake of Cr^{51} measured over the liver and spleen.

4. *Red Cell Survival.*—Fig. 1 shows the results obtained by injecting J. B. with Cr^{51}-labeled group A, V positive, Rh negative cells. His own cells, tagged with P^{32}, serve as a control. It can be seen that about 50 per cent of the incompatible cells disappeared from the peripheral blood within 20 minutes. Thereafter, the rate gradually decreased, so that less than 30 per cent of the injected radioactivity remained after one hour. There was a simultaneous uptake of Cr^{51} activity measured over the spleen, suggesting that this organ was the major site of red cell destruction. Considerably less radioactivity was measured over the area of liver under the scintillation counter, where a small decline in the slope occurred. The fact that this decrease in counts did not parallel that in the peripheral blood probably indicates some red cell destruction in the liver.

Fig. 2 indicates the localization of the patient's own cells labeled with Cr^{51}. During the one-hour period of the experiment, there was no significant disappearance of the injected cells from the peripheral blood. The concentration of radioactivity in both the spleen and liver did not vary significantly after the initial rise.

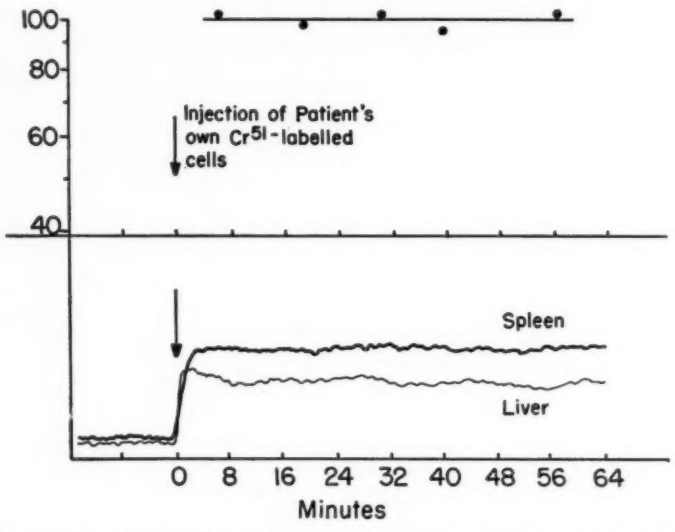


Fig. 2.—Above: Percentage of injected radioactivity in peripheral blood (log scale). Below: Radioactive uptake of Cr^{51} measured over the liver and spleen.

DISCUSSION

The results presented in this paper confirm the previously reported high incidence of the V antigen in Negroes as well as its occurrence only in cells containing the gene combination ce on the same chromosome.

The fact that the three examples of anti-V so far encountered have occurred in individuals whose cells were Rh negative (with respect to the D antigen) is contrary to expectation and would appear to be more than coincidental. Assuming that the only factor preventing the possible formation of anti-V is the presence of the V antigen, by far the most numerous candidates for making this antibody should be Rh positive, V negative recipients of Rh positive, V positive blood. No such examples have been found, in spite of an intensive search in our laboratory and by Dr. Cahan (personal communication). To explain this discrepancy at least two possibilities may be considered. First, the V antigen, like C, may rarely cause an antibody response in Rh (D) positive recipients. If this were true, one would expect to find the antibody in those Rh negative, V negative individuals who had been sensitized by the relatively uncommon blood from an Rh negative V positive donor (or baby, in the case of pregnancy). Alternatively, Rh negative, V negative recipients of the much more common Rh positive, V positive blood could also form the antibody. This was very probably the case in the two examples encountered in this laboratory.

A second possibility deserving consideration is that anti-V has, in fact, been found in the blood of Rh positive subjects, but not recognized as such. For example, a serum containing anti-c or anti-e, and/or anti-f would agglutinate all cells containing the V antigen, because these cells must contain c, e, and (usually) f. It would therefore be necessary to absorb any such serum with rr (cdef/cdef) V negative cells in order to reveal the presence of anti-V. To our knowledge, no studies of this sort have been carried out.

The rarity of this antibody renders its clinical importance very small. However, red cell survival studies reported in this paper show that the antibody is capable of destroying cells in vivo, and might therefore be responsible for hemolytic transfusion reactions. The V positive red cell destruction curve shown in Fig. 1 is not unlike that reported by Mollison and Cutbush[3] for anti-D and anti-Kell, both of which (like the J. B. anti-V) usually behave in the antiglobulin test as gamma globulins. Furthermore, the rapid localization of susceptible cells in the spleen is similar to that observed by Jandl[5] when D positive cells coated with anti-D were injected. For these reasons, it would appear necessary to consider the possibility of anti-V as the causative agent in unexplained cases of transfusion reaction and hemolytic disease of the newborn.

SUMMARY

The presence of anti-V in the serum of two previously transfused patients is reported. The ability of this antibody to destroy red cells in vivo was demonstrated in one of these patients.

Racial studies confirmed the previously reported high incidence of the V antigen in Negroes and its rare occurrence in white subjects. The antigen was also found to be unusual in orientals and American Indians.

Further confirmation was obtained for the hypothesis that the V gene is present only on chromosomes with the Rh genes c and e. The occurrence of V with cDe is apparently more frequent than with cde.

The possible significance of the fact that the three examples of anti-V so far discovered have occurred in Rh (D) negative individuals is discussed.

We are indebted to Drs. R. R. Race, Ruth Sanger, and Amos Cahan for confirming some of the data presented in this paper. We also wish to thank Mrs. Millie Russell and Miss Lucy Brooks for technical assistance.

REFERENCES

1. DeNatale, A., Cahan, A., Jack, J. A., Race, R. R., and Sanger, Ruth: V, a "New" Rh Antigen, Common in Negroes, Rare in White People, J. A. M. A. **159**: 247-250, 1955.
2. Dacie, J. V.: The Haemolytic Anemias, Congenital and Acquired, New York, 1954, Grune and Stratton, p. 237.
3. Mollison, P. L., and Cutbush, Marie: Use of Isotope-Labelled Red Cells to Demonstrate Incompatibility in Vivo, Lancet **1**: 1290-1295, 1955.
4. Cutbush, Marie, Crawford, Hal, and Mollison, P. L.: Observations on Anti-human Globulin Serum, Brit. J. Haemat. **1**: 410-421, 1955.
5. Jandl, J. H.: Sequestration by the Spleen of Red Cells Sensitized With Incomplete Antibody and With Metallo-Protein Complexes, J. Clin. Invest. **34**: 912, 1955.

THE PERSISTENCE IN THE BLOOD OF THE RADIOACTIVE LABEL OF ALBUMINS, GAMMA GLOBULINS, AND GLOBULINS OF INTERMEDIATE MOBILITY

IV. Specific Activity Ratios Between Plasma and Edema Fluid of I^{131}-Labeled Albumin Injected Intravenously in Nutritional and Nephrotic Hypoproteinemia

D. S. Kushner, M.D., D. Bronsky, M.D., A. Dubin, M.S., B. P. Maduros, M.D., and S. H. Armstrong, Jr., M.D.
Chicago, Ill.

INTRODUCTION

THE last year has seen the publication of two careful studies[1, 2] in which plasma protein fractions labeled in vitro with I^{131} have been used toward the solution of this classic problem: Is the hypoproteinemia of the nephrotic state due simply to urinary loss, or is there a further pathologic defect in protein metabolism?

Valid interpretation of the data presupposes, of course, that the intravenously injected iodoprotein is handled in the same fashion as is the nephrotic subject's own autosynthesized proteins.

Therefore the following statement in the paper of Gitlin, Janeway, and Farr[2] becomes of great interest: "direct examination . . . revealed that in the period of exponential decline of iodoalbumin in the plasma, the specific activity of albumin in the edema fluid, in counts per minute per gram of albumin, was roughly 30 times that of the plasma."

Many workers have recognized that moderate differences in specific radioactivity of an I^{131}-iodoprotein from simultaneously drawn pleural fluid, ascitic fluid, and plasma may relate to differences in rates of equilibration between the respective body compartments and the blood stream. The thirtyfold difference found by Gitlin and his co-workers lies far outside the realm of the moderate. If differences of this sort could be regularly demonstrated, they would invalidate the method of estimation of "total exchangeable pool" of a protein which has been customary since Sterling's[3] initial studies on I^{131}-iodoalbumin; namely, by the extrapolation of the logarithmic biologic decay plot to the time of protein administration in order to find the specific activity of the body pool, had the injected protein immediately and uniformly become distributed throughout it.

From the Hektoen Institute for Medical Research of the Cook County Hospital and the Departments of Medicine of the University of Illinois College of Medicine and Northwestern University Medical School.

These studies have been specifically supported by Grant A-927 from the National Institute of Arthritis and Metabolic Diseases of the United States Public Health Service.

Received for publication Aug. 23, 1956.

In an adult nephrotic patient chosen from the group under long-term study at the Cook County Hospital we have therefore undertaken the following observations: (a) the comparison of albumin specific radioactivities in plasma, urine, and edema fluid in the metabolic die-away period following intravenous I^{131} albumin; (b) comparison of the apparent turnover times of albumins from these three sources; (c) the comparison of the extrapolation estimates of total exchangeable albumin pool before and after the intravenous injection over some two weeks of unlabeled normal human serum albumin[4] in amount greater than ten times the subject's estimated initial total exchangeable albumin pool; (d) the comparison of plasma and urinary albumin apparent turnover times before and after the massive albumin infusions. (Comparison of edema albumin turnover times was not possible as the patient proved to be one of the nephrotics who became edema-free at the end of the infusions.)[5]

Because there is much to suggest common factors to both nephrotic and nutritional edema, we also have compared specific radioactivities and apparent turnover times of plasma and edema fluid albumin in two patients whose hypoproteinemia was comparable in degree to our nephrotic subject but entirely of nutritional origin.

PATIENTS AND METHODS

Selection of Patients.—

Adult nephrotic syndrome: J. McC.'s histologic diagnosis of chronic membranous glomerulonephritis had been made by renal biopsy about a year before the study here reported. Suffering from intractable nephrotic edema over this year, she has had repeated observations of the electrophoretic distributions of her plasma and urinary proteins as well as the customary clinical and biochemical studies. These make her comparable as an adult to the children reported by Gitlin and co-workers under the designation of Group III.[2]

Nutritional edema: T. C., a man 70 years of age, has been under continued observation by our gastrointestinal and metabolic groups for some nine months prior to this study. Repeated radiologic, hematologic, and biochemical workups had disclosed no other basis for his hypoproteinemia than a continuous mild disorientation related to arteriosclerosis, absence of dentures, and an apathy to eating both the basic hospital diet and all supplements when not directly forced. His nutritional edema has never been very extensive.

W. S. (likewise under observation by our metabolic group for over a year before this study) had been subjected to repeated surgery for peptic ulceration at another hospital. When admitted to Cook County Hospital his edema amounted to anasarca. Though temporarily and partially responsive to intensive nutritional therapy, his course was disturbed by many bouts of subacute intestinal obstruction. At the time of this study there was no evidence of obstruction but his edema was again very extensive.

Subsequent to this study intravenous human serum albumin failed to halt a downhill course. Autopsy disclosed ubiquitous intestinal adhesions and an anastomosis such that food passed directly from remains of stomach to terminal ileum.

Isotope Administration, Dosage, and Counting.—At each labeling the patient received 200 μc of I^{131}-human serum albumin intravenously.

Both patients with nutritional edema received Abbott* Lot No. 1078-52-65. The nephrotic subject on initial labeling received Abbott Lot No. 1078-106-36. These lots are representative of this laboratory's standard preparation without special precautions for long-term metabolic work.[6]

For the second labeling of the nephrotic subject, Abbott Lot No. 1045-39-1 was obtained by courtesy of Dr. D. Tabern. Following iodination this labeled albumin was diluted with an excess of normal human serum albumin on the basis of the storage self-radiation injury suggested for I^{131}-insulin by Berson and co-workers.[7]

A twenty-minute serum sample was used for estimation of plasma volumes since recent evidence has supported the validity of this timing after the I^{131}-albumin injection even in the presence of anasarca.[8] Thereafter serum, urine, and edema samples were obtained and studied as described below at intervals indicated in the figures to follow. Lugol's solution was given during the studies.

Radioactivity measurements in well-type scintillation counters were corrected for radioactivity decay to the time of intravenous injection of the labeled protein.

Edema Fluid Protein.—

Drawing edema fluid: Edema fluid was obtained through skin punctures made with a sharp No. 18 needle. As each drop appeared it was sucked into a dry tuberculin syringe through a No. 23 needle. Thus the fluid was obtained from the surface of the skin and not by the insertion of the withdrawing instrument into the subcutaneous spaces.

An experience of many years in obtaining edema fluid has led to a preference for this procedure over that of Southey tubes, particularly when edema is minimal, and has also led to these precautions: (a) if the initial skin puncture perforates a venule and bleeding persists for more than a couple of minutes, this puncture is abandoned in order to avoid any significant serum contamination; (b) pressure on the subcutaneous tissues to speed the expression of edema fluid must be made with the fingers of the hands at least two inches from the puncture, otherwise the final fluid obtained may have a slightly higher protein content than that of the initial fluid. While this difference has not in our experience amounted to more than 20 mg. per cent, for purposes of this study such a difference is inadmissable.

Preparation for paper electrophoresis: Edema fluid presents to a lesser degree problems in preparation for electrophoresis familiar to those who have worked with spinal fluid. If the protein content is 0.2 Gm. per cent or less, the direct application of as much as 100 λ to the paper strip will lead to a diagram so faint as to be difficult to scan photometrically,† and also falsely high in the globulin components and falsely low in the albumin.

The often-used procedure of exposing a cellophane bag of edema fluid to the draft of an electric fan at either room temperature or at +5° C., while effective in dehydrating the contents, can lead to unreliable electrophoretic and ultracentrifugal results on some proteins thus concentrated. The well-known surface denaturation of proteins when dried from aqueous solution is accentuated when the starting solution is as dilute in protein as is edema fluid (thus yielding a very low ratio of protein mass to cellophane surface area). Also, the concentration method concentrates electrolytes, etc., as well as protein.

Recently, we have been concentrating our edema fluid by the method used by de la Huerga[9] for spinal fluids. A minimum of 1 ml. (if possible, 2 ml.) of edema fluid are put in a cellophane bag and dialyzed at +5° C. against 50 ml. of 20 per cent Vinisil.‡

An overnight dialysis usually removes all visible fluid from within the bag.

If this bag is removed from the Vinisil and stored in the atmosphere until the time for the setting up of the electrophoretic run, and the dried protein removed therefrom

*Abbott Laboratories, North Chicago, Ill.

†Electrophoretic measurements in this study have been carried out with Spinco Model R Durrum-type cell; photoelectric intergration has been carried out on the Spinco Analytrol, Beckman Instrument Co., Belmont, Calif.

‡Polyvidone prepared by the Abbott Laboratories, North Chicago, Ill.

by dissolving it in about 0.25 ml. of buffer, the resultant electrophoretic patterns show to varying degrees a sharp artifact at the starting boundary. A photograph both of a paper electrophoretic pattern and the resultant photoelectric scanning illustrating this artifact is given in Fig. 1. The artifact is caused by a failure of part of the applied protein to migrate in the electric field, again related to surface denaturation. While the artifact can be corrected for by subtracting its area from the gamma area in the photoelectric scanning, it can be avoided as follows.

The cellophane bag containing the edema fluid is dialyzed by day with careful observation until only about a drop appears to remain within the bag. The bag is then immediately removed from the Vinisil and is not allowed to dry further through contact with air. The drop is immediately removed by pipette and 100 lambda are applied directly to the electrophoretic paper without any addition of buffer.

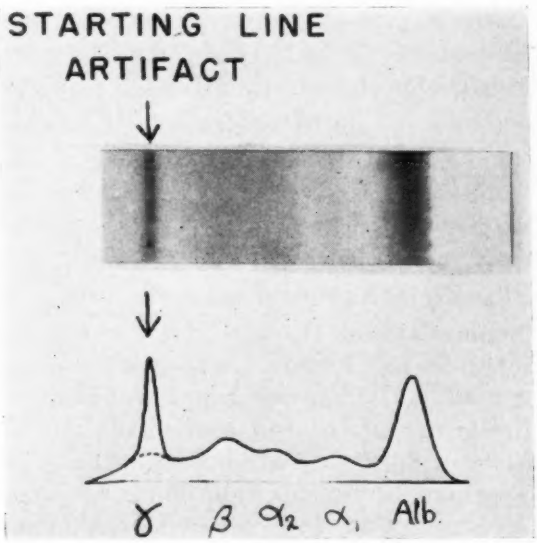


Fig. 1.—For description, see text.

While it is recognized that free electrophoresis yields gamma globulin values somewhat lower than those obtained by paper electrophoresis,[10] the avoidance of denaturation keeps further error to a methodological minimum.

Urinary Proteins.—The high protein content of J. McC.'s urine did not necessitate the above procedure nor the others available for protein concentration in the presence of lesser degrees of proteinuria.[11, 12]

Twenty-four-hour collections of urine were made over dry ice in a portable refrigerated unit placed at the patient's bedside. By this method each voiding was immediately frozen and so stored until time for study. On thawing, 100 ml. was filtered through Whatman No. 4 paper and the pH adjusted to 5.4 with concentrated pH 4 acetate buffer used in Method 10.[13] Ninety-five per cent ethanol prechilled to 0° C. was then slowly added, the temperature being maintained throughout below +5° C. The mixture was then brought to −5° C. The protein precipitate, spun down at this temperature in a refrigerated centrifuge, was redissolved in some 10 ml. of diethylbarbiturate buffer pH 8.6, ionic strength 0.1, and dialyzed against 100 times its volume of this buffer overnight at 0° C.

The solution of urinary proteins was then quantitatively washed out of the dialysis bag. The volume was accurately recorded. The protein concentration was refractometrically measured as previously described by one of us.[14] This solution was then used for electrophoretic and other measurements.*

A considerable experience with this procedure has led us to believe that the losses in pipetting, transferring, centrifugation, etc., amount to less than 20 per cent of the total protein present and are not localized to any one electrophoretic component.

RESULTS AND DISCUSSION

Protein Distributions and Electrophoretic Patterns.—The three patients of this study all had comparable total serum proteins of 4 Gm. per cent or below and comparable serum albumin levels of about 1 Gm. per cent. They differed significantly in total cholesterol. J. McC.'s elevation to 870 mg. per cent was characteristic of the nephrotic state on an unrestricted diet. T. C.'s 143 mg. per cent and W. S.'s 73 mg. per cent represent the depression usually seen in hypoalbuminemia of nutritional restriction.

In Fig. 2 are shown the initial electrophoretic scannings of the serum, urine concentrate, and edema protein concentrate of J. McC., the nephrotic patient. In Fig. 3 these are shown for T. C. (mild nutritional edema); in Fig. 4, for W. S. (severe nutritional edema). Subsequent serial scanning did not differ significantly for the latter patients, and for J. McC. changed in the expected fashion[5] following the massive albumin infusions.

The chief difference between the sera of the nutritional edema subjects and those of the nephrotic is the well-known elevation of the $alpha_2$ and beta peaks of the latter condition. This elevation is not proportionately reflected in either the nephrotic urinary protein concentrate nor in the edema fluid concentrate. The edema fluid concentrates of all subjects have somewhat higher relative proportions of albumins and lower of gamma globulins than do the corresponding sera. While these differences in proportional concentrations can be arranged in order of the average molecular weights of the respective electrophoretic components,[15] they probably cannot be wholly explained thereby.

All edema fluids show total protein contents well within the low range characteristic of nephrotic edema (which in our experience rarely exceeds 0.4 Gm. per cent, save after massive intravenous albumin therapy, and then rarely exceeds 1 Gm. per cent).

By way of contrast is shown the electrophoretic scanning of serum and edema in a patient, D. J., with a general defect in systemic capillary permeability (Fig. 5). While this patient died before isotopic studies could be carried through, the data illustrate the virtual identity both in total protein content and distribution of electrophoretic components of his serum and of the fluid of his general anasarca.

*For determination of total urinary radioactivity excretions, and total urinary protein radioactivity excretions, scintillation counter measurement was made on an aliquot of the 24-hour specimen. Saturated with $(NH_4)_2SO_4$, the aliquot is then boiled, the total accurately diluted to 2.5 times the original aliquot volume, and filtered, and scintillation counter measurements are made on the protein-free filtrate.

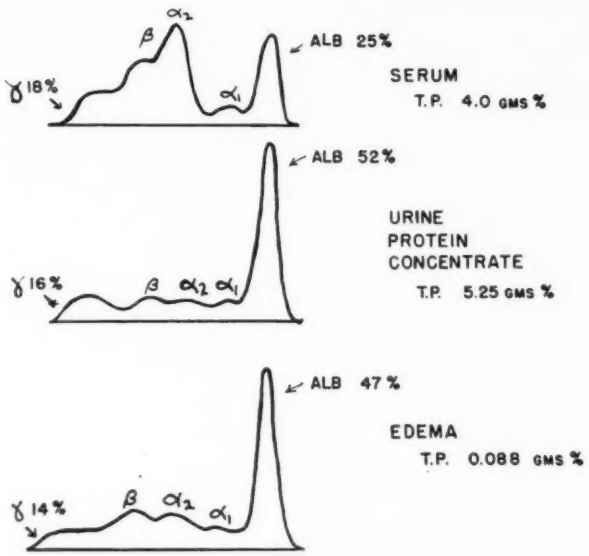


Fig. 2.—Patient J. McC. Adult nephrotic syndrome. (See text for description.)

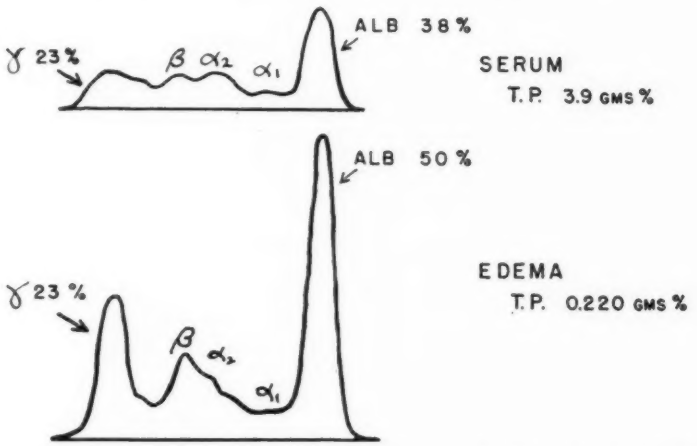


Fig. 3.—Patient T. C. Mild nutritional edema. (See text for description.)

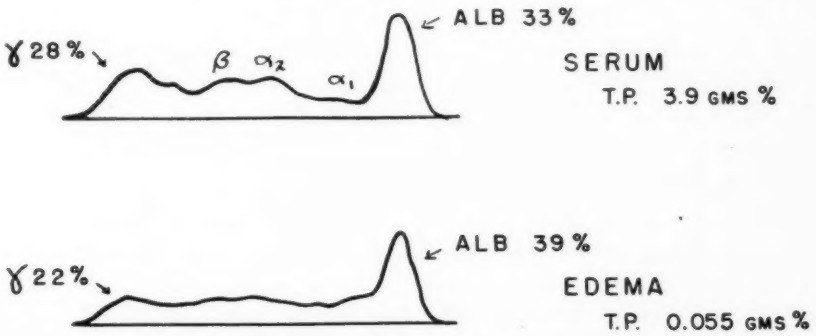


Fig. 4.—Patient W. S. Severe nutritional edema. (See text for description.)

Apparent Turnover Times of Albumin Component.—The corrected radioactivity data are plotted for purposes of illustration on an arbitrary scale in Figs. 6 to 10. This is to permit spatial separation of the decay plots for the albumins of various sources.

The *apparent* turnover times ($T_{½}/1n2$) for the serum albumins of all three subjects are markedly shortened as compared with a normal control studied contemporaneously and with our own and other workers' previous normal figures (Table I).

TABLE I

PATIENTS		APPARENT TURNOVER TIMES (DAYS)		
		SERUM ALBUMIN	URINE ALBUMIN	EDEMA ALBUMIN
J. McC. (Nephrotic)	initial	7	6	7
	after 825 Gm. alb. i.v.	8	6	–
T. C. (Nutritional, mild)		7	–	7
W. S. (Nutritional, severe)		12	–	18
J. M. (Normal control)		17	–	–

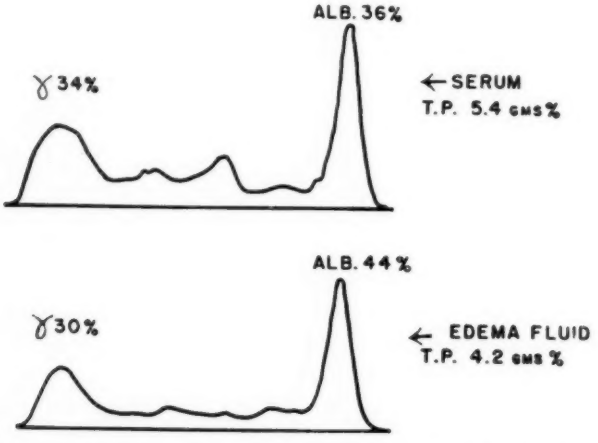


Fig. 5.—Patient D. J. Erythema multiforme with increased systemic capillary permeability. (For description, see text.)

In Fig. 6 are shown the die-away plots for serum, urine, and edema albumin of J. McC. (nephrotic) before the massive intravenous albumin therapy. The apparent turnover times (calculated from the slopes of these plots) are essentially identical; the difference of one day between the plasma and urinary albumin lies too close to the combined experimental errors of the method to be interpreted.

The die-away plots shown in Fig. 7 follow a second intravenous injection of I^{131}-serum albumin. This injection was made one week after the end of the massive intravenous albumin therapy (825 Gm. in 17 days). The interval of one week was allowed in order that the closest approximation to the steady state possible in this situation might be achieved. By this time her serum albumin levels had stabilized at about 1.5 Gm. per cent higher than the

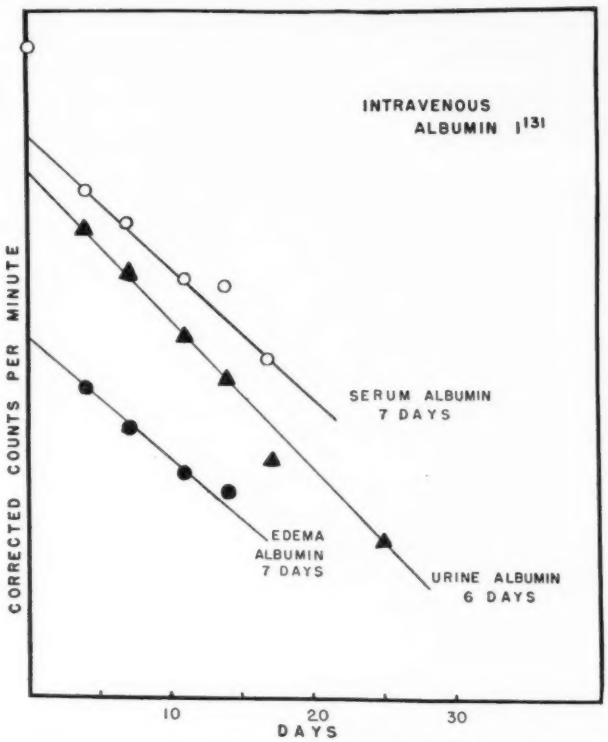


Fig. 6.—Patient J. McC. Adult nephrotic syndrome moderate edema. (For description, see text)

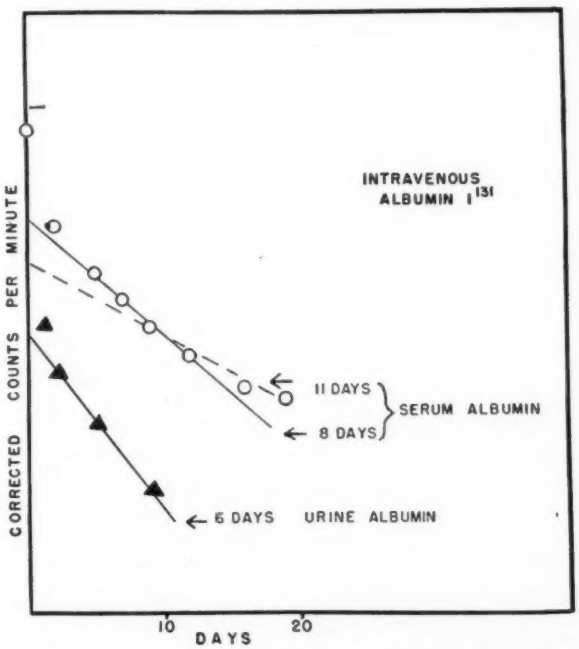


Fig. 7.—Patient J. McC. Adult nephrotic syndrome after massive human albumin therapy (For description, see text.)

pretherapy level. That this approximation to the steady state is not directly comparable to the situation following the labeling in Fig. 6 is indicated by contrasting these representative 24-hour figures:

	TOTAL PROTEINURIA GM./24 HR.	ALBUMINURIA GM./24 HR.
Before therapy	13 ± 2	7 ± 1
After therapy	22 ± 2	14 ± 1

No edema data are shown in Fig. 7, for the therapy was correlated with a weight loss of some 30 pounds and the disappearance of visible edema.

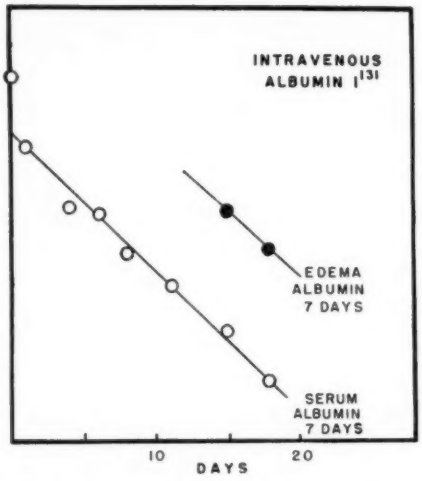


Fig. 8.—Patient T. C. Mild nutritional edema. (For description, see text.)

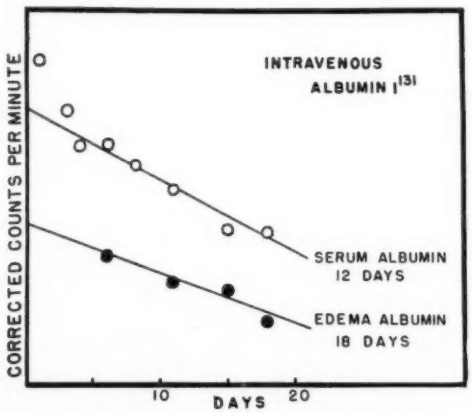


Fig. 9.—Patient W. S. Massive nutritional edema. (For description, see text.)

While the apparent turnover time for urinary albumin appears uninfluenced by the therapy, the apparent turnover time for serum albumins has become slightly prolonged when estimated from the die-away plot's slope between days 5 and 12. Moreover, the die-away plot shows some terminal flattening between days 12 and 21. Not often seen following intravenous

I^{131}-proteins, terminal flattening has already been discussed with respect to S^{35} proteins[19]; it is important in the discussion of exchangeable pool estimates to follow.

The die-away plots of T. C. (mild nutritional edema) and W. S. (severe nutritional anasarca) are given in Figs. 8 and 9, respectively.

The former patient shows no difference in apparent turnover time between serum and edema albumin. The latter shows a 6-day difference which is well beyond experimental error and which indicates that the albumin in W. S.'s anasarca is turning over at a significantly slower rate than is his serum albumin.

Comparison of Specific Activity Ratios.—In Fig. 10 specific activity ratios are plotted against time. All data are from samples taken after a five-day "equilibration" period has elapsed after injection of the labeled protein.

The ratios between serum, urine, and edema albumin can be seen to lie close to unity for J. McC. This finding for edema albumin is very different from the findings of Gitlin and co-workers[2] for childhood nephrotics. For urinary albumins it was confirmed on J. McC. following the second labeling and is in essential agreement with the findings of Blahd and co-workers[1] for adult nephrotics.*

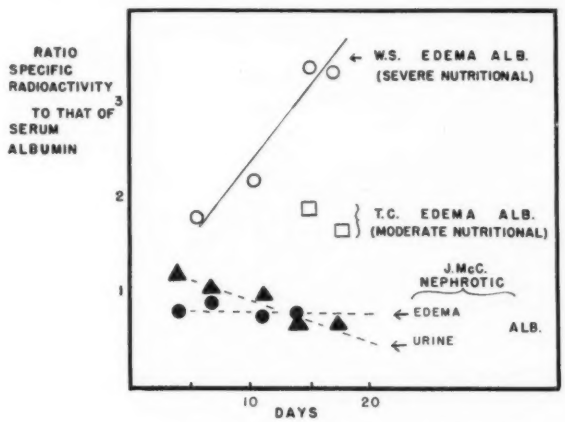


Fig. 10.—For description, see text.

For T. C.'s edema albumin the specific activity ratio to serum albumin appears greater than unity but less than two. For W. S. the ratio starts in T. C.'s range and increases such that by the end of two weeks it is well over three. While three is a small figure by comparison with Gitlin and co-workers' thirty, if representative of all extravascular albumin, it could lead to serious error in total exchangeable pool estimation.

The explanation most readily at hand is that in the presence of very massive anasarca the isotopically labeled albumin that diffuses from capillary into edema fluid is to some extent trapped there and is not subject to the

*The downward slope of the dotted line in Fig. 10 for J. McC.'s urinary proteins was not found on the occasion of her second labeling. Here the ratio of urinary albumin specific activity to that of plasma albumin approximated unity with no directional trend of time.

metabolic processes characteristic of the albumin remaining in the circulation. This is another way of saying that equilibration fails. Such trapping could readily account for the more prolonged turnover time and rising specific activity of W. S.'s edema albumin as compared to his serum albumin.

Estimates of Total Exchangeable Albumin Pools.—Estimates made by extrapolation of the die-away plot *after* the equilibration period to zero time (as described in the introduction) are obviously valid only to the extent that equilibration is complete* While the evidence of the previous section indicates that in Patient W. S., at least, this is not the case, we have carried out such estimates using for extrapolation the slopes between day 5 and day 12. The results, together with quantities derived by application of the apparent turnover times of Table I, are given for all three patients in Table II together with results for the contemporaneously studied normal control subject, J. M.

TABLE II

PATIENTS		PLASMA VOLUME (ML.)	"APPARENT FINAL VOLUME ALBUMIN DISTRIBUTION" (ML.)	TOTAL EXCHANGEABLE ALBUMIN POOL (GM.)	TURNOVER GM./DAY	TURNOVER %/DAY
J. McC. (Nephrotic)	initial	2,600	6,780	60	9	14
	after 825 Gm. alb. i.v.	3,400	9,420	275	34	12
T. C. (Nutritional, mild)		2,400	4,350	58	8	14
W. S. (Nutritional, severe)		2,250	4,540	45	4	8
J. M. (Normal control)		----	6,700	215	13	6

Total exchangeable albumin pools for J. McC. (nephrotic) prior to therapy and for both patients with nutritional edema appear reduced to about a quarter of the pool estimated for the normal control subject. The smallest pool (45 Gm.) was estimated for W. S.

The massive intravenous albumin therapy apparently restored J. McC.'s total exchangeable pool to normal. But if a balance sheet is constructed, a quantitative discrepancy emerges.

INITIAL POOL (1ST LABELING)		60 GM.
Injected albumin	825 Gm.	
minus		
Totaled increments in albuminuria during therapy and before second labeling	460 Gm.	
Albumin added to pool by therapy		365 Gm.
Pool after therapy { Predicted		425 Gm.
Pool after therapy { Found (second labeling)		275 Gm.
Discrepancy		150 Gm.

No evidence for increased "burning" of the injected albumin could be found in the urinary nonprotein nitrogen output either during the therapy or in the second die-away period.

*If injected labeled protein were initially *concentrated* extravascularly beyond the theoretical equilibrium value, this would lead to a falsely high pool estimate. If, however, equilibrium were not reached initially and the extravascular labeled protein were subsequently trapped in a fashion inaccessible to metabolic processes for intravascular protein, this would lead to a falsely low pool estimate.

Nor does a ready explanation emerge from the hypothesis that J. McC.'s artificially swollen albumin pool is not in the "steady state" but is slowly decreasing during the die-away period of the second labeling because of the greater albuminuria.

For a progressively decreasing pool will lead to an *overestimate* of total exchangeable pool and a *lengthening* of apparent turnover time by flattening the die-away plot. While the albumin therapy has brought about the latter effect (a lengthening of some 10 per cent) with respect to the pool, we are faced with an experimental *underestimate* of the predicted pool by some 35 per cent.

If we use the slope of the die-away plot between days 12 to 21 (Fig. 7, dotted line) rather than between days 5 to 12 (Fig. 7, solid line) to extrapolate for zero time intercept, the observed terminal flattening results in a total exchangeable pool estimate of some 400 Gm. The discrepancy between predicted and estimated pool thus disappears. But the question arises: By what right should one choose the terminally flattened slope rather than the slope during the customary metabolic die-away period for the estimate?*

So when we use the figures of the last column of Table II in order to compare the pre- and posttherapy *endogenous* metabolism of albumin, we are faced with a potential error that is of the order of magnitude of the figures we are trying to compare.

Thus, for J. McC.'s albumin:

	BEFORE THERAPY	AFTER THERAPY
Total turnover (Gm./day)	9	34
Urinary loss (Gm./day)	7	14
Metabolic turnover (Gm./day)	2	20
"Fractional metabolic turnover rate"[2] (%/day)	3	7

The last pair of figures might lead to the conclusion that J. McC. had a fractional metabolic albumin turnover rate doubled by massive albumin therapy (and brought close to the figure for the normal control [J. M., Table II]) were it not so clear that a decrease of but 1 Gm. a day in pretherapy albuminuria could correspond to a nearly *doubled* rate.

Gitlin and co-wokers'[2] Equation 1 gives an estimate of daily albumin synthesis which does not depend on extrapolation of a die-away plot to zero time. For the posttherapy labeling of J. McC., the fraction of the total labeled dose lost in the urine at the end of the twenty-one day experiment was 60 per cent. This leads to an estimated daily albumin synthesis of 24 Gm., a figure less by one-third than that obtained from the slope and extrapolated intercept of the die-away plot (Fig. 6, solid line).

*After the posttherapy labeling of J. McC., the ratio of nonprotein to protein urinary radioactivity followed this course:

Day 1	27 per cent
Day 2	54 per cent
Day 4	41 per cent
Day 5	30 per cent
Day 9	30 per cent
Day 12	16 per cent
Day 16	15 per cent

The increase in proportion of degraded isotope excreted in the mixing period (days 1 to 5), as well as the subsequent few days, might suggest that the injected labeled protein contained components undergoing metabolic degradation at different rates. It still remains an assumption to take the final rate as characteristic of the total nonlabeled albumin pool.

If we substitute 24 Gm. per day for 34 Gm. per day in the right-hand column above, we might conclude that J. McC's fractional metabolic turnover rate has been uninfluenced by the massive intravenous therapy.

However, we do not believe figures from these two methods are comparable with sufficient precision to validate either conclusion.

SUMMARY

In three patients with hypoalbuminemic edema (one nephrotic, two nutritional) the specific activity of edema albumin has been compared with that of contemporaneously obtained serum albumin (and for the nephrotic's urine albumin) following intravenous injection of I^{131}-labeled albumin.

Apparent turnover times of albumins from all sources were found markedly shortened; total exchangeable pools were reduced to about a quarter of the normal range.

Edema fluid albumin specific activity ratio to serum (and for the nephrotic urine) albumin did not deviate strikingly from unity in two patients. In the third (massive nutritional anasarca), an edema fluid albumin apparent turnover time longer than for serum, and a specific activity ratio rise to over three in a fortnight, was taken to indicate a failing of equilibration between extra and intravascular albumin, and thus a trapping of some of the labeled protein beyond the reach of the metabolic processes to which serum albumin is subject.

In the nephrotic patient, comparison was made of total exchangeable pool size before and after massive intravenous albumin therapy (825 Gm.). While the initially depleted pool was apparently restored to normal, the increment proved significantly less than the increment predicted from the dose and the urinary protein and nitrogen loss.

These findings are discussed in terms of some of the interpretative pitfalls involved in apparent turnover time and exchangeable pool estimates from extrapolation of radioactivity die-away plots, particularly in patients who may deviate somewhat from the "steady state."

We are indebted to the Drs. Leonard H. and Louis D. Weissman Medical Research Foundation for the backing of the studies of plasma proteins as related to disease mechanisms at the Hektoen Institute for Medical Research of the Cook County Hospital. We are also indebted to Warden Fred Hertwig of the Cook County Hospital for his continued interest in the development of clinical investigation at the Cook County Hospital.

REFERENCES

1. Blahd, W. H., Fields, M., and Goldman, R.: The Turnover Rate of Serum Albumin in the Nephrotic Syndrome as Determined by I^{131}-Labeled Albumin, J. LAB. & CLIN. MED. **46**: 747, 1955.
2. Gitlin, D., Janeway, C. A., and Farr, L. E.: Studies on the Metabolism of Plasma Proteins in the Nephrotic Syndrome. I. Albumin, Gamma Globulin and Iron-Binding Globulin, J. Clin. Invest. **35**: 44, 1956.
3. Sterling, K.: The Turnover Rate of Serum Albumin in Man as Measured by I^{131}-tagged Albumin, J. Clin. Invest. **30**: 1228, 1951.
4. Cohn, E. J., Oncley, J. L., Strong, L. E., Hughes, W. L., Jr., Armstrong, S. H., Jr.: Chemical, Clinical, and Immunological Studies on the Products of Human Plasma Fractionation. I. The Characterization of Protein Fractions of Human Plasma, J. Clin. Invest. **23**: 417, 1944.

5. Thorn, G. W., Armstrong, S. H., Jr., Davenport, V. D., Woodruff, L. M., and Tyler, F. H.: Chemical, Clinical, and Immunological Studies on the Products of Human Plasma Fractionation. XXX. The Use of Salt-poor Concentrated Human Serum Albumin Solution in the Treatment of Chronic Bright's Disease, J. Clin. Invest. **24**: 802, 1945.
6. Tabern, D.: Personal communication.
7. Berson, S. A., Yalow, R. S., Bauman, A., Rothschild, M. A., and Newerly, K.: Insulin I^{131} Metabolism in Human Subjects: Demonstration of Insulin Binding Globulin in the Circulation of Insulin Treated Subjects, J. Clin. Invest. **35**: 170, 1956.
8. Moir, T. W., Pritchard, W. H., and Ford, A. B.: The Early Disappearance of I^{131} Serum Albumin From the Circulation of Edematous Subjects and Its Implications in the Clinical Determination of the Blood Volume, J. LAB. & CLIN. MED. **47**: 503, 1956.
9. de la Huerga, J.: Personal communication.
10. Mackay, I. R., Volwiler, W., Goldsworthy, P. D., Eriksen, N., and Wood, P. A.: Paper Electrophoresis of Serum Proteins: Photometric Quantitation and Comparison With Free Electrophoresis, J. Clin. Invest. **33**: 855, 1954.
11. McGarry, E., Sehon, A. H., and Rose, B.: The Isolation and Electrophoretic Characterization of the Proteins in the Urine of Normal Subjects, J. Clin. Invest. **34**: 832, 1955.
12. Sellers, A. L., and Marmorston, J.: Electrophoretic Study of Human Urinary Protein in Disease, J. LAB. & CLIN. MED. **47**: 248, 1956.
13. Cohn, E. J., Gurd, F. R. N., Surgenor, D. M., Barnes, B. A., Brown, R. K., Derouaux, G., Gillespie, J. M., Kahnt, F. W., Lever, W. F., Liu, C. H., Mittelman, D., Mouton, R. F., Schmid, K., and Uroma, E.: A System for the Separation of the Components of Human Blood: Quantitative Procedures for the Separation of the Protein Components of Human Plasma, J. Am. Chem. Soc. **72**: 465, 1950.
14. Armstrong, S. H., Jr., Budka, M. J. E., Morrison, K. C., and Hasson, M.: Preparation and Properties of Serum and Plasma Proteins. XII. The Refractive Properties of the Proteins of Human Plasma and Certain Purified Fractions, J. Am. Chem. Soc. **69**: 1747, 1947.
15. Oncley, J. L., Scatchard, G., and Brown, A.: Physical-chemical Characteristics of Certain of the Proteins of Normal Human Plasma, J. Phys. & Colloid Chem. **51**: 184, 1947.
16. Schoenberger, J. A., Kroll, G., Eckert, E. L., and Kark, R. M.: Investigation of Transfer Rates of Albumin Tagged With I^{131} in Ascites and Edema. II. Studies in Control Subjects and Patients With Cirrhosis, J. LAB. & CLIN. MED. **47**: 227, 1956.
17. Bauer, F. K., Blahd, W. H., and Fields, M.: Ascitic Fluid and Plasma Protein Exchange. Studies With Radioiodinated Human Serum Albumin, J. Clin. Invest. **32**: 553, 1953.
18. Berson, S. A., Yalow, R. S., Schreiber, S. S., and Post, J.: Tracer Experiments With I^{131} Labeled Human Serum Albumin: Distribution and Degradation Studies, J. Clin. Invest. **32**: 746, 1953.
19. Armstrong, S. H., Jr., Bronsky, D., and Hershman, J.: The Persistence in the Blood of the Radioactive Label of Albumins, Gamma Globulins and Globulins of Intermediate Mobility. III. Comparison of Die-away Plots Following Oral and Intravenous Administration of the S^{35} Label to the Same Subjects, J. LAB. & CLIN. MED. **46**: 857, 1955.

LABORATORY METHODS

A COLORIMETRIC METHOD FOR TRANSAMINASE IN SERUM OR PLASMA

W. W. UMBREIT,* PH.D., RAHWAY, N. J., G. R. KINGSLEY,** M.S., R. R. SCHAFFERT,** A.B., LOS ANGELES, CALIF., AND HERMAN SIPLET,*** A.B., PHILADELPHIA, PA.

LADUE[1-3] and co-workers have described a relationship between the level of serum transaminase and myocardial infarction. This increase in enzyme apparently holds for experimental myocardial infarction in dogs and in acute myocardial infarction in man. The basic observations have been widely confirmed and discussed[4-11] so that this relationship may be a useful diagnostic tool. It has been suggested recently that similar determinations might serve as an index of hepatocellular integrity. The methods usually employed for measurements of serum transaminase are spectrophotometric and involve the use of auxiliary enzyme preparations (usually malic dehyrogenase). They offer no difficulties in laboratories equipped to handle them, but the methods suffer from a restriction in their use, particularly in many laboratories in which the equipment and specialized skills are not available.

A colorimetric method can be derived from an earlier method proposed by Tonhazy, White, and Umbreit[12] which employs the reaction between aspartic and α-ketoglutarate, and measures the dinitrophenylhydrazone of pyruvate formed from the chemical decarboxylation of oxalacetate. Since the absorption spectrum of this substance is so broad, essentially any colorimeter may be used and any of wide-range wave lengths are suitable (450 to 520 mμ may be used). In order to be used on serum, the method of Tonhazy and co-workers[12] required some modification. Modifications found suitable in practice are described. The method devised was validated by use under practical conditions in hospital laboratories and by comparison with other methods.

METHOD

Reagents.—The reagents are similar to those described by Tonhazy, White and Umbreit.[12]

Reagent A: Transaminase substrate. 2.66 Gm. DL-aspartic acid; 2 Gm. K_2HPO_4, 0.6 Gm. α-ketoglutaric acid, dissolved, adjusted with KOH to pH 7.4 and made to 100 ml. Subject to bacterial attack; should be refrigerated.

Reagent B: 100 per cent trichloracetic acid. 100 Gm. solid trichloracetic acid made to 100 ml.

From the Merck Institute for Therapeutic Research, Rahway, N. J.,* Veterans Administration Center, Los Angeles 25, Calif.,** and the Fels Research Institute, Temple University School of Medicine, Philadelphia 40, Pa.***

Received for publication June 16, 1956.

Reagent C: Aniline citrate. 5 Gm. citric acid plus 5 ml. H_2O; when dissolved add 5 ml. aniline. This may crystallize in time; when it can no longer be handled, it should be discarded.

Reagent D: Dinitrophenylhydrazine reagent. 100 mg. 2,4-dinitrophenylhydrazine dissolved in 20 ml. concentrated HCl and 80 ml. H_2O. With time this reagent tends to give increasing colors in blank determinations. When these interfere, fresh reagent should be employed.

Reagent E: Water-saturated toluene. 5 ml. water shaken with 500 ml. toluene.

Reagent F: Alcoholic KOH. 2.5 Gm. KOH dissolved in 100 ml. 95 per cent alcohol. If this reagent absorbs CO_2 from the air it may become turbid. It should therefore be protected as much as convenient from CO_2, and when turbid should be replaced by fresh reagent.

PROCEDURE

1. Serum or plasma (citrated) plus other additions or water to 1.0 ml. (usually 0.5 ml. serum or plasma is used).
2. At zero time add 0.5 ml. Reagent A.
3. At 10 minutes add 1 drop Reagent B, 1 drop Reagent C. (These two reagents cannot be mixed.) Shake, allow to stand for at least 20 minutes. The determination can be interrupted at this point indefinitely.
4. Then add 0.5 ml. Reagent D; shake, stand for 5 minutes (this must be timed; 4.5 to 5.5 minutes satisfactory).
5. Add 2 ml. Reagent E and shake vigorously. Centrifuge to break any emulsion which may be formed. It is sometimes necessary to break up with a stirring rod a thick layer formed on the first centrifuging, so that adequate amounts of a clear supernate can be obtained. Unless this is done recovery of pyruvate may be poor.
6. Remove 1 ml. Reagent E layer and put into colorimeter tube.
7. Add 3 ml. Reagent F, shake, measure color at a constant wave length between 450 to 520 mμ.

BASIS AND VALIDITY OF METHOD

The reaction employed in this method is the following:

$$\text{aspartate} + \alpha\text{-ketoglutarate} \rightarrow \text{oxalacetate} + \text{glutamate}$$
$$\downarrow$$
$$\text{pyruvate} + CO_2$$

The reaction (catalyzed by the enzyme transaminase) proceeds for a definite time in the presence of excess aspartate and α-ketoglutarate (Reagent A). At the end of this time (usually 10 minutes) the reaction is stopped (Reagent B) and the oxalacetate formed decomposed chemically to pyruvate and CO_2 (Reagent C). The pyruvate thus formed is allowed to react with dinitrophenylhydrazine (Reagent D) and the dinitrophenyl hydrazone formed is extracted by toluene thus separating it from the excess reagent and the α-ketoglutarate dinitrophenylhydrazone which may have formed since the latter is essentially insoluble in toluene. This is based on a method previously described by Friedemann and Haugen.[13] The pyruvate dinitrophenylhydrazone in the toluene layer is converted to a colored compound by treatment with the strong alkali in alcohol in which the toluene remains in solution.

There are in blood (and sometimes in other tissues), materials which will be extracted by the toluene and form-colored complexes in alkali. These materials, whatever their nature, do not extract in the presence of Reagent A (or in the presence of α-ketoglutarate equivalent to that supplied by Reagent

A). Therefore, the usual type of control, i.e., elimination of one and of both of the components of the reaction mixture, will not apply to blood serum or plasma. Under these circumstances proof that the system is measuring transaminase consists of evidence that the rate of reaction is proportional to time and to concentration of blood serum, and that the method agrees with other methods not subject to this limitation. In addition, it can be shown that added purified transaminase can be estimated.

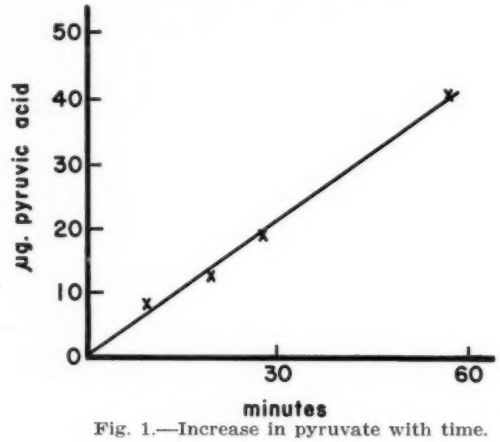


Fig. 1.—Increase in pyruvate with time.

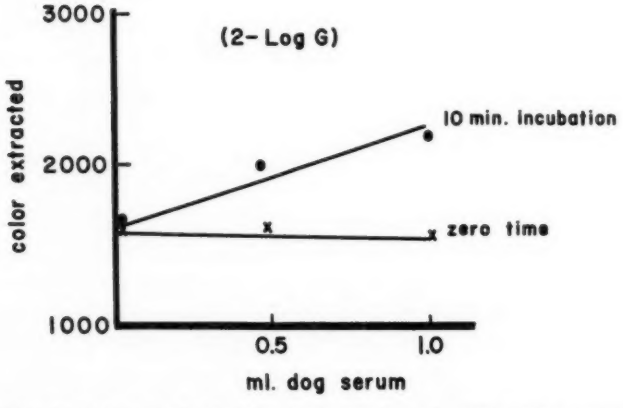


Fig. 2.—Increase in color on incubation of serum is proportional to amount of serum.

Fig. 1 provides evidence that the increase in pyruvate is a straight-line function with time over the period of an hour. Fig. 2 shows that increase in color (pyruvate formed) on incubation with dog serum is proportional to the amount of serum. It also demonstrates that the color extractable at zero time is that due to α-ketoglutarate in the Reagent A (zero milliliter dog serum), and that the presence of this reagent prevents extraction of other chromogens from the serum itself.

The unit of transaminase activity as defined by LaDue and associates[1-3] is that amount which causes a decrease of 0.001 in optical density at 340

mμ in one minute in the malic dehydrogenase enzyme system. If the total volume of the system is 3 ml., this would be equivalent to the formation of 0.475 μg pyruvate per ten minutes in the system described in this paper. The rate of transaminase activity will vary with the temperature so that it is necessary to assume that the LaDue unit is defined at 37° C. Therefore, one unit of transaminase activity is equivalent to the formation of 0.475 μg pyruvate per ten minutes in this standard system. One milliliter dog serum produced in the standard system, 15.9 μg pyruvic acid, or showed 15.9/0.475 = 33.4 units per milliliter while 0.5 ml. dog serum produced 6.95 μg pyruvic acid or showed 14.7 units equivalent to 29.4 units per milliliter. The 0.5 ml. serum used in Fig. 3 contained 15 units, equivalent to 30 units per milliliter. These values of about 30 units per milliliter agree well with those reported by LaDue and co-workers.[1-3]

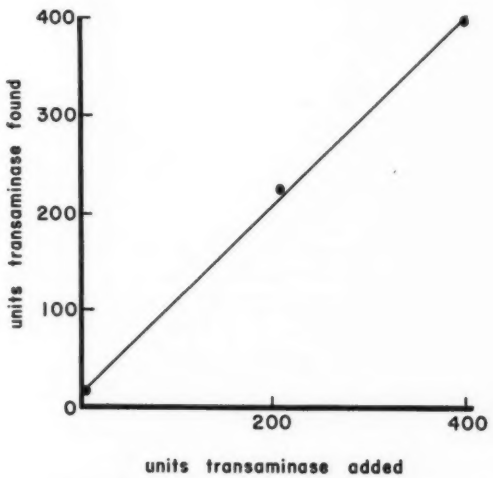


Fig. 3.—Detection and estimation of transaminase in serum.

Apparently the normal human sera contains 30 to 40 units.[1-3] The data above show that this amount is detectable, the increase in optical density being something of the order of 0.03 to 0.05, depending on the type of colorimeter, and other equipment, employed. These are low, but measurable, values. A day after myocardial infarction[1-3] the units of transaminase rise to 100 to 6,000 units per milliliter. The essential problem here would be: Could this colorimetric method detect such an increase? For this purpose we prepared a cell-free transaminase from rat heart containing 4,000 units per milliliter. Fig. 3 shows that small amounts of transaminase are readily detected and quantitatively recovered, and the method could detect thus easily the increases reported following myocardial infarction. The differences in color between 30 units transaminase and 200 units are so striking as to be readily visible to the naked eye.

A comparison has been made between the method proposed by Karmen[14] which has an accuracy of about ±10 transaminase units and a slight modification of the method described above on 196 patients. The data, and operating details, given in Table I, show that the agreement is quite good. In general the experience has been that values above 60 to 80 units are obtained with patients having myocardial infarcts and that values below these levels are not seen in such patients except toward the end of the recovery period.

TABLE I. COMPARISON METHODS FOR SERUM TRANSAMINASE

UNITS BY KARMEN METHOD	NUMBER OF CASES	UNITS FOUND IN UMBREIT METHOD (μG. PYRUVATE/ML./MIN.) 0.0475	
		RANGE	AVERAGE
10	26	11- 40	22
20	69	6- 57	36
30	24	8- 76	52
40	13	32- 63	52
50	10	61-107	73
60	4	70- 89	80
80	10	101-145	119
90	5	133-162	140
100-140 (115)*	7	120-194	161
140-190 (160)	9	166-225	192
200-210 (205)	5	225-289	264
260-800 (450)	4	252-527	362

*Average of group.

Modification Employed: Na_2HPO_4 was used instead of K_2HPO_4 in the buffered aspartate. Incubation was carried out for fifteen minutes instead of ten minutes. 0.5 ml. of 10 per cent, instead of 0.1 ml. of 100 per cent trichloracetic acid was used. Final centrifugation was carried out routinely for separation of toluene extract. In order to obtain suitable volume to read color in a Coleman photometer, 5 ml. of 2.5 per cent KOH in 95 per cent alcohol was added instead of 3 ml. A maximum absorption band at 450 mμ was obtained with Coleman photometers. These studies were done by Kingsley and Schaffert at the Veterans Administration Center, Los Angeles, Calif.

In another laboratory, a slightly different modification was employed (for details see Table II) and serum samples from 212 normal individuals averaged 24.1 ± 6.18 units while 11 samples from patients with myocardial infarcts resulted in values exceeding 3 times the standard deviation. Samples were exchanged between two laboratories using different methods and the data of Table II indicate a satisfactory practical agreement.

TABLE II. COMPARISON OF INDIVIDUAL DETERMINATIONS DONE BY DIFFERENT METHODS IN DIFFERENT LABORATORIES

PATIENT	UNITS TRANSAMINASE/ML. SERUM	
	COLORIMETRIC METHOD	SPECTROPHOTOMETRIC METHOD
1	15	18
2	77.2	75.0
3	52.8	30.0
4	62.5	60.0
5	32.8	35.0
6	44.0	15.0
7	199	102
8	155	75

The colorimetric method consisted of 0.1 ml. serum + 0.9 phosphate buffer and after the addition of 0.5 ml. of Reagent A, incubated thirty minutes at 25° C. in a water bath. The subsequent procedure is as described in this paper except that 5 ml. of Reagent F is used and after mixing 1 ml. of water is added, reading at 490 in an Evelyn colorimeter. Transaminase units = 800 (net optical density + 0.007). These experiments were done by Siplet at the Fels Research Institute, Philadelphia, Pa. The spectrophotometric determinations were done by the method of Karmen and associates,[2] by Dr. J. H. Boutwell of Temple University Hospital Laboratories.

SUMMARY

A colorimetric method for transaminase suitable for serum is described. This has been tested under clinical conditions and found to be satisfactory.

REFERENCES

1. LaDue, J. S., Wroblewski, F., and Karmen, A.: Serum Glutamic Oxalacetic Transaminase Activity in Human Acute Transmural Myocardial Infarction, Science **120**: 497, 1954.
2. Karmen, A., Wroblewski, F., and LaDue, J. S.: Transaminase Activity in Human Blood, J. Clin. Invest. **34**: 126, 1955.
3. LaDue, J. S., and Wroblewski, F.: Significance of SGO-Transaminase Activity Following Acute Myocardial Infarction, Circulation **11**: 871, 1955.
4. Steinberg, D., and Ostrow, B. H.: Serum Transaminase as a Measure of Myocardial Necrosis, Proc. Soc. Exper. Biol. & Med. **89**: 31, 1955.
5. Nydick, I., Wroblewski, F., and LaDue, J. S.: Evidence of Increased Serum Glutamic Oxalacetic Transaminase (SGO-T) Activity Following Graded Myocardial Infarcts in Dogs, Circulation **12**: 161, 1955.
6. Agress, C. M., Jacobs, H. I., Glassner, H. F., Lederer, M. A., Clark, W. G., Wroblewski, F., Karmen, A., and LaDue, J. A.: Serum Transaminase Levels in Experimental Myocardial Infarction, Circulation **11**: 711, 1955.
7. Chinsky, M., Shmagranott, G. L., and Sherry, S.: Serum Transaminase Activity. Observations in a Large Group of Patients, J. LAB. & CLIN. MED. **47**: 108, 1956.
8. Goldner, F., and Meador, C.: Transaminase and C-Reactive Protein Levels in Acute Myocardial Infarction: Evaluation of Indices of Necrosis, South. Med. J. **48**: 1339, 1955.
9. McHardy, R. J.: Clinical Evaluation of Serum Transaminase Levels in Myocardial Infarction, South. Med. J. **48**: 1347, 1955.
10. Kattus, A. A., Watanabe, R., Semenson, C., Drell, W., and Agress, C.: Serum Aminopherase (Transaminase) in Diagnosis of Acute Myocardial Infarction, J. A. M. A. **160**: 16, 1956.
11. Molander, D. W., Wroblewski, F., and LaDue, J. S.: Serum Glutamic Oxalacetic Transaminase as an Index of Hepatocellular Integrity, J. LAB. & CLIN. MED. **46**: 831, 1955.
12. Tonhazy, N. E., White, N. G., and Umbreit, W. W.: A Rapid Method for the Estimation of the Glutamic-Aspartic Transaminase in Tissues and Its Application to Radiation Sickness, Arch. Biochem. **28**: 36, 1950.
13. Friedemann, T., and Haugen, G. E.: Pyruvic Acid. II. The Determination of Keto Acids in Blood and Urine, J. Biol. Chem. **147**: 415, 1943.
14. Karmen, A.: A Note on the Spectrophotometric Assay of Glutamic-Oxalacetic Transaminase in Human Blood Serum, J. Clin. Invest. **34**: 131, 1955.

THE RELATIONSHIP BETWEEN BACTERIOPHAGES OF SALMONELLAE AND THEIR O ANTIGENS

JOHN H. SILLIKER, PH.D., AND WELTON I. TAYLOR, PH.D.
CHICAGO, ILL.

BACTERIOPHAGES which were species or even strain specific in their lytic activity were reported by d'Herelle.[1] Certain other bacteriophages showed ability to attack a number of species within a given genus. Raettig[2-3] refers to the latter phages as polyvalent, whereas d'Herelle called them heterologous; we have chosen to designate them wide spectrum phages. One such agent possessing wide spectrum activity was the bacteriophage strain O-1, isolated by Felix and Callow.[4] Cherry and associates[5] reported this phage to demonstrate a most catholic ability to lyse salmonellae and they used this characteristic as a means of generic identification.

Wasserman and Saphra[6] isolated bacteriophages against individual species in the various serologic groups within the genus Salmonella. Their investigations on the activity of those bacteriophages led them to conclude that, with few exceptions, a bacteriophage active against one organism within a serologic group will likewise show lytic activity against other members of the same group, but not against other serotypes lacking somatic antigens in common with the original host strain. They concluded that the ability of a bacteriophage to attack different salmonellae bears a direct relationship to the O antigens of the organism. The results of an investigation of a similar nature is presented herewith for comparison with those data and conclusions.

MATERIALS AND METHODS

A transfer of bacteriophage O-1 and its parent host *Salmonella paratyphi B* was obtained. Cherry and co-workers[5] had reported that approximately one-half of the salmonellae resistant to this phage belonged to somatic Group E; accordingly 6 species representing that group were obtained. A phage spectrum was determined and among the refractory strains were *S. chittagong, S. muenchen (oregon), S. illinois,* and *S. newington.* Bacteriophages active against each of these species were isolated from sewage aeration mixture (raw sewage and returned sludge).* Tests for bacteriophage activity were made using the method of Craigie and Yen,[7] and subsequent determination of antisalmonellae activity of these four phages revealed them to be wide spectrum phages. Both 1 per cent tryptone nutrient agar and brilliant green agar were used. The degree of lysis varied occasionally with the medium used, and brilliant green agar was eventually selected as the medium of choice for determination of lysis.

From the Research Laboratories, Swift & Company, Chicago 9, Ill.
Received for publication June 27, 1956.

*Obtained through the courtesy of the Southwest Sewage Treatment Plant of the Sanitary District of Chicago.

RESULTS

Data obtained using five wide spectrum phages against over 250 cultures of Salmonella species were carefully examined for evidence of a correlation between phage spectra and the O antigens possessed by parent strains. We were unable to discover any such correlation. Our results, in fact, lead away from such a conclusion. The indications are, rather, that: (1) a bacteriophage may attack one or more strains of a given Salmonella species and be completely unable to attack other strains of that species (Table I), (2) a phage may fail to attack many species which have the same somatic antigens as its parent strain, *e.g.*, the failure of O-1 phage to attack some other group B species (Tables II and III), (3) a phage may attack species having no somatic antigens in common with either the parent strain or each other (Table III),

TABLE I. PHAGE SPECTRA FOR DIFFERENT STRAINS OF THE SAME SALMONELLA SPECIES DEGREE OF LYSIS BY BACTERIOPHAGE*

SPECIES	STRAIN	O-1 PHAGE	S. CHITTAGONG PHAGE [(1), III, X, (XIX)]	S. NEWINGTON PHAGE (III, XV)
S. montevideo (Group C_1, VI, VII)	2	++	–	–
	22	+	+	+
	25	+++	–	–
	26	+	–	–
	28	+	–	–
	7,511	–	+	–
S. bareilly (Group C_1, VI, VII)	5	++	+	–
	24	–	–	+
	7,411	–	++	–
	7,504	++	–	++++
	8,441	–	+	+++
S. anatum (Group E_1, III X, XXVI)	11	–	++++	+
	30	–	+	++++
	2,917	+	++	++++

*++++ indicates no resistant clones, – no plaques.

TABLE II. ACTION OF PHAGE O-1 AGAINST GROUP B ORGANISMS

SALMONELLA SPECIES (GROUP B)	BACTERIOPHAGE O-1 (DEGREE OF LYSIS)
S. paratyphi B (parent), (I, IV, V, XII)	++++
S. derby, (I, IV, XII)	–
S. typhimurium, 20, (I, IV, V, XII)	–
S. typhimurium, 8,411 (I, IV, V, XII)	++++
S. bredeney, (I, IV, XXVII, XII)	++++
S. st. paul, (I, IV, V, XII)	++++
S. chester, (IV, V, XII)	++++
S. reading, (IV, XII)	++++

TABLE III. ACTION OF PHAGES ON SPECIES WITH LIKE AND UNLIKE O ANTIGENS OF THE PARENT STRAINS

GROUP	STRAINS	DEGREE OF LYSIS BY PHAGES	
		O-1	S. OREGON (C_2) (VI, VIII)
C_1 (VI, VII)	*S. thompson*	++++	++
	S. montevideo, 2	++	+
	S. montevideo, 25	+++	+
	S. oranienburg	+++	++
	S. bareilly, 7,504	++	–
	S. bareilly, 5	++	–
	S. mikawasima	–	++
	S. tennessee	–	++
	S. infantis	+++	++
C_2 (VI, VIII)	*S. newport*	+++	++
	S. oregon	–	++++
	S. manchester	++++	+++

TABLE IV. DISSIMILARITIES BETWEEN THE SPECTRA OF PHAGES FROM PARENTS OF LIKE O ANTIGENS

GROUP	SPECIES	SOMATIC ANTIGENS	DEGREE OF LYSIS BY PHAGES	
			S. ILLINOIS (E_2) ([III], [XV])	S. NEWINGTON (E_2) (III, XV)
B	*S. typhimurium*	I, IV, V, XII	–	+
C_1	*S. bareilly*, 7,504	VI, VII	–	++++
C_1	*S. oranienburg*, 21	VI, VII	+	–
C_2	*S. newport*	VI, VIII	+	++++
E_1	*S. london*	III, X, XXVI	–	+++
E_2	*S. illinois*	(III), (XV)	++++	–
E_2	*S. newington*	III, XV	–	+++
E_3	*S. chittagong*	(I), III, X, (XIX)	–	++

and (4) two phages isolated for two parents having identical O antigens do not react with each other's parents, nor are they similar in their spectra toward other species of either like or unlike O antigens (Table IV).

DISCUSSION

The failure to show consistent and reproducible correlation between phage susceptibility and the possession of similar O antigens, as demonstrated by the data presented, raises considerable doubt as to the existence of such a relationship. It was reported by d'Herelle[8] that phages active against single strains of *S. typhosa* existed, and indeed epidemiological data upon typhoid fever is routinely gathered by utilizing such phages in England.[4] Atkinson and Geytenbeek[9] demonstrated both narrow and wide spectrum phages of *S. adelaide* among their isolates from lysogenic strains. They characterized their phages as Types 1 and 2 and observed Type 1 to be active exclusively against *S. adelaide* strains, while Type 2 was active against almost all other salmonellae. They were surprised to find that three strains of *Escherichia coli*, 0111, were not lysed by any of their phages since Olarte and Varela[10] had shown *S. adelaide* and that coliform to have Salmonella O antigen XXXV

in common. Cherry and co-workers[5] similarly reported the failure of O-1 phage to attack 13 strains of Salmonella species having O antigen XXXV. In Table I it may be seen, for example, that five strains of *S. bareilly* vary in their susceptibility to the phage of *S. newington,* from completely negative in two strains to complete lysis in one with two strains showing a lesser degree of lysis.

Failure of a wide spectrum phage to attack other species having antigens in common with the parent strain is demonstrated in Table II in which *S. derby* and *typhimurium* resist the action of O-1 phage, and in Table III, two strains of *S. bareilly* are refractory to lytic action of *S. oregon* phage. Both Wasserman and Cherry found a strain of *S. derby* to resist B group phage, and Atkinson's *S. adelaide* phages did not lyse three strains of *Escherichia coli,* 0111, which have the identical O antigen.

That phages will attack species lacking any antigens in common is borne out by the existence of every wide spectrum phage. Mallman[11] utilized concomitant growth of *Shigella dysenteriae* and *Salmonella pullorum* in mixed culture to adapt a phage specific for the former to parasitism of both. Raettig[3] reported that phage Pb-So, originally specific for *S. paratyphi B* and used since 1925, now lyses a variety of enteric organisms including *Shigella,* coliform, and other Salmonella species. He interprets the change from specific to nonspecific to be a maturation process. This is demonstrated in Table III in which O-1 phage is as effective against Groups C_1 and C_2 species as is the wide spectrum phage of *S. oregon* (Group C_2).

In Table IV one observes that two phages isolated against parents of like O antigens do not react with each other's parents, nor do they have the same spectra for other species. This may be explained by postulating that each is the lysogenic phage of the other's parent strain; thus the *S. newington* phage is actually the lysogenic phage which accompanied *S. illinois* parent, and vice versa. This phenomenon is also observed by Atkinson with phages of *S. adelaide,* notably with the relationship between phage Types 1a and 1b.

It is interesting to note that the strains of phages isolated by Wasserman and Saphra reacted so well against other species of their parent groups. Seemingly, *S. derby* is recalcitrant to Group B phages regardless of the investigator or the phage used (Table II, Cherry and associates and Wasserman and Saphra). The ability of D group phage of Wasserman to lyse B group species bespeaks the type of cross reactions that we have found to be the rule with our phage isolates rather than the exception. Much of our work was performed on Salmonella species upon original isolation from nature rather than upon stock cultures of known organisms. Our results with phage were known long before the cultures were identified by the Public Health Laboratory. The result of our phage investigations have led us to pool our five phages and use them for rapid identification of the genus *Salmonella* (unpublished data) without attempting to type by group with phage. We have executed a similar

survey of Bethesda-Ballerup and Arizona paracolons and found wide spectrum phages which are universal in their lytic ability against these organisms, but which do not lyse either coliform or salmonellae (unpublished data), and they exhibit total disregard for both the O and H antigens involved.

SUMMARY

The results obtained in typing over 250 newly isolated Salmonella strains with wide spectrum bacteriophages exhibit a pronounced failure to conform to any schema of Salmonella species susceptibilities based on O antigens possessed by phage parent strains. The consistent occurrence of cross reactions among these phages is discussed and illustrated.

We wish to gratefully acknowledge the help of the State of Illinois Public Health Laboratories, Chicago, for their assistance in obtaining O-1 phage and Salmonella cultures, and for typing strains isolated from nature.

REFERENCES

1. d'Herelle, F.: The Bacteriophage and Its Behavior (Translated by G. H. Smith), Baltimore, Md., 1926, Williams and Wilkins Co.
2. Raettig, H.: Experimentelle Untersucher über Entwicklungsvorgäne bei Bakteriophagen. II. Die polyvalenten Bakteriophagenstämme in Darmschleimen von Mensch und Tier, Zentralbl. Bakt. Abt. I. **158**: 34-53, 1952.
3. Raettig, H.: V. Mitteilung. Die Entwicklung von neuen Valenzen und Typen bei Diagnostik-Phagenstammen, Zentralbl. Bakt. Abt. I. **161**: 11-38, 1954.
4. Felix, A., and Callow, B. R.: Typing of Paratyphoid B Bacilli by Means of Vi Bacteriophage, Brit. Med. J. **2**: 127-130, 1943.
5. Cherry, W. B., Davis, B. R., Edwards, P. R., and Hogan, R. B.: A Simple Procedure for the Identification of the Genus Salmonella by Means of a Specific Bacteriophage, J. Lab. & Clin. Med. **44**: 51-55, 1954.
6. Wasserman, M. M., and Saphra, I.: The Use of Bacteriophages in Typing Salmonella Cultures, J. Bact. **69**: 97-100, 1955.
7. Craigie, J., and Yen, C. H.: The Demonstration of Types of B. Typhosus by Means of Preparation of Type II Vi Phage, Can. J. Pub. Health **29**: 448-463, 1938.
8. d'Herelle, F.: Du role du microbe filtrant bacteriophage dans la fievre typhoide, Compt. Rend. Acad. Sc. **168**: 631, 1919.
9. Atkinson, N., and Geytenbeek, H. G.: Salmonella bacteriophages. I. Bacteriophages of S. adelaide, Australian J. Exper. Biol. & M. Sc. **31**: 441-452, 1953.
10. Olarte, J., and Varela, G.: A Complete Somatic Antigen Common to Salmonella adelaide, Escherichia coli—Gomez and Escherichia coli 0111:B4, J. Lab. & Clin. Med. **40**: 252-254, 1952.
11. Mallman, W. L.: Studies on Bacteriophage in Relation to Salmonella and Pullorum Disease, Agr. Exper. Station, Mich. State Coll., Tech. Bull. **109**: 1-15, 1931.

PHOTOELECTRIC METHOD FOR ESTIMATION OF THE OXYGEN SATURATION OF NONHEMOLYZED WHOLE BLOOD

PAUL SEKELJ, D. ENG., AND ARNOLD JOHNSON, M.D.
MONTREAL, QUEBEC

VARIOUS spectrophotometric and photoelectric methods have been described for the in vitro determination of the oxygen saturation in whole blood.* In recent years the interest in these methods has been greatly increased by the demand for immediate estimation of the oxygen saturation of blood samples withdrawn at the time of cardiac catheterization. In view of the difficulty of in vitro determinations under anaerobic conditions when the red cells are intact, methods of estimating the oxygen saturation in hemolyzed blood or in diluted hemoglobin solutions have been developed.[1-5] Although these techniques may approach the accuracy of the manometric methods, the time required for the necessary manipulation of the samples limits their usefulness under some conditions. The purpose of this paper is to describe the operation and principles of construction of an apparatus which provides a rapid estimation of the oxygen saturation of small quantities of whole blood flowing at a constant rate through a cuvette.

PRELIMINARY EXPERIMENTS

Extensive trial experiments were carried out to re-examine certain aspects of the light transmission through thin layers of stationary and flowing blood. These experiments were purely qualitative in nature, their aim being to observe the interdependence of some factors influencing the light transmission in this system.

1. The reproducibility and the accuracy of the determinations are dependent on the rate of blood flow through the cuvette. Different shapes of the cuvette and various depths of 0.045, 0.07, and 0.10 cm. have been tried and the rate of flow has been varied within wide limits. Measurements have been carried out on dog's and cow's blood, on samples taken from discarded, but still fresh, human bank blood, and on aliquots of human blood obtained during cardiac catheterization.

These studies permit the following conclusions: (a) utilization of highly diffused light apparently reduces the effect of light scatter; (b) the depth of the cuvette in the selected range, at identical rates of flow, does not significantly affect the determinations; (c) the rate of flow must exceed some limiting value

From the Department of Physiology, McGill University and Department of Cardiology, Montreal Children's Hospital.

Aided in part by grants from the National Research Council of Canada and by the Playtex Park Research Institute.

Received for publication July 16, 1956.

*The literature on this subject has been extensively reviewed by E. H. Wood in Otto Glasser's Handbook of Medical Physics, Vol. II, 1950.

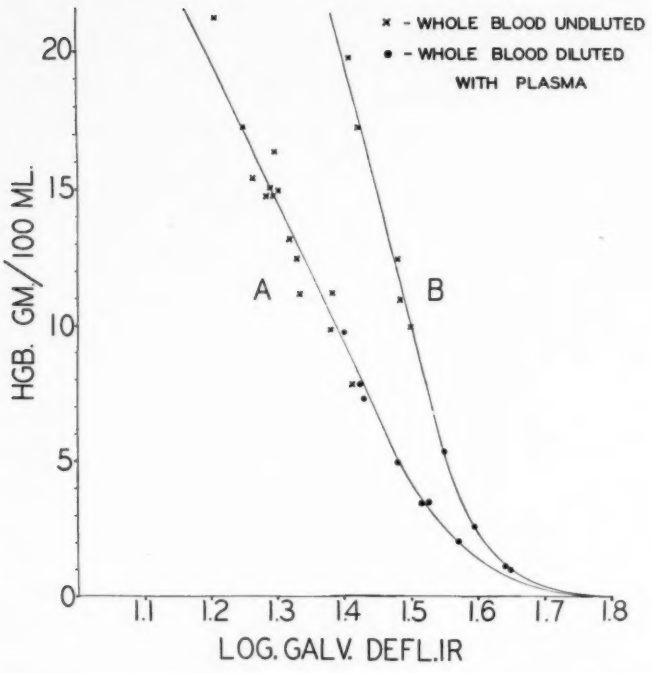


Fig. 1.—Relationship between the logarithm of the total hemoglobin concentration and the logarithm of the photocurrent with the infrared filtered photocell. Blood stationary in the cuvette. *A*, Depth 0.07 cm.; *B*, depth 0.045 cm.

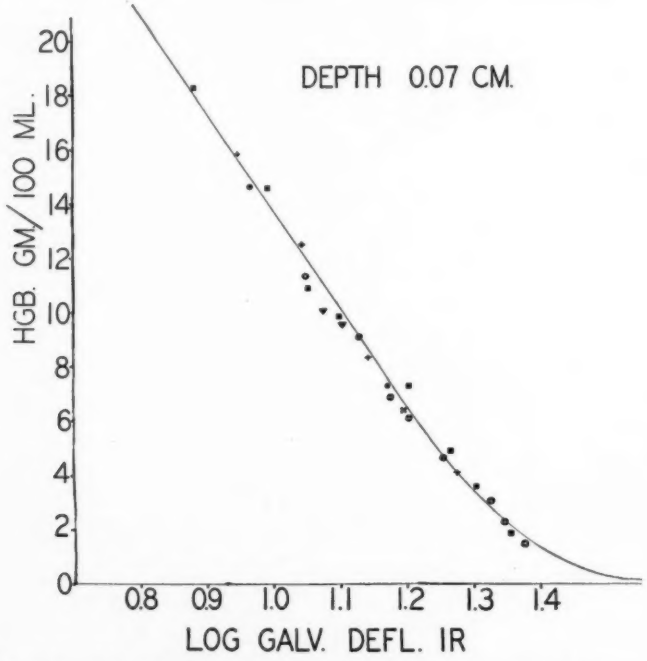


Fig. 2.—Relationship between the logarithm of the total hemoglobin concentration and the logarithm of the photocurrent with the infrared filtered photocell. Blood flowing through the cuvette.

in order to obtain the steady galvanometer readings necessary for the desired high degree of reproducibility of successive determinations; (d) the amount of transmitted light increases with a decrease in the rate of flow and asymptotically approaches a plateau when the blood is stationary in the cuvette, depending upon the oxygen saturation, the erythrocyte concentration, and the sedimentation rate.

The pilot studies resulted in the choice of a cuvette of 0.07 cm. depth and in a rate of flow of 4 ml. per minute. At this rate of flow the average velocity of the blood flowing through the Lucite cuvette (illustrated in the insert of Fig. 3) is about 0.45 cm. per second.

2. It has been shown in a previous paper[7] that the measurements in the infrared region with the photocell covered by 2 or 3 layers of Wratten No. 87 filter are independent of the blood oxygen saturation. The observed variability in the infrared readings over the whole range of oxygen saturation from 0 to 100 per cent was of the order of 2 per cent, i.e., within the limit of the accuracy of the measurements.

3. Kramer and associates[8] have demonstrated that if the erythrocyte concentration does not change and if monochromatic light is used for transillumination, a linear relationship exists between the oxyhemoglobin concentration and the logarithm of the light transmission. They have further shown that the Beer-Lambert law of absorption does not hold for the total hemoglobin concentration of whole blood but that the relationship between the hemoglobin concentration and the logarithm of the light transmission (galvanometer deflection) is nonlinear. We have made similar observations. These are illustrated in Figs. 1 and 2. In these graphs the hemoglobin concentration in grams per 100 ml., derived from oxygen capacity values determined by the Sendroy method,[9] is plotted against the logarithm of the galvanometer deflection with the infrared filtered photocell. Curves *A* and *B* in Fig. 1 refer to measurements with the blood stationary in the cuvette, and the curve in Fig. 2 refers to measurements with the blood flowing through the cuvette at a constant rate of 4 ml. per minute. The samples for these experiments were obtained from different subjects during cardiac catheterization and were used undiluted and/or diluted with the blood's own plasma. With the blood stationary in the cuvette, readings were taken at 30-second intervals during 240 seconds and then averaged. The differences in the contour of the curves of Figs. 1 and 2 are due to the fact that the experimental conditions and the photocells used were different.

APPARATUS AND OPERATION

The oximeter used for these studies and the circuitry and mode of operation are the same as described previously.[6, 7] Operation of a double-scale ear or double-scale whole blood oximeter may be selected by means of a selector switch. The only difference in the circuitry consists of an additional variable resistance R_4 (ref. 7: Fig. 3, page 1749) in the

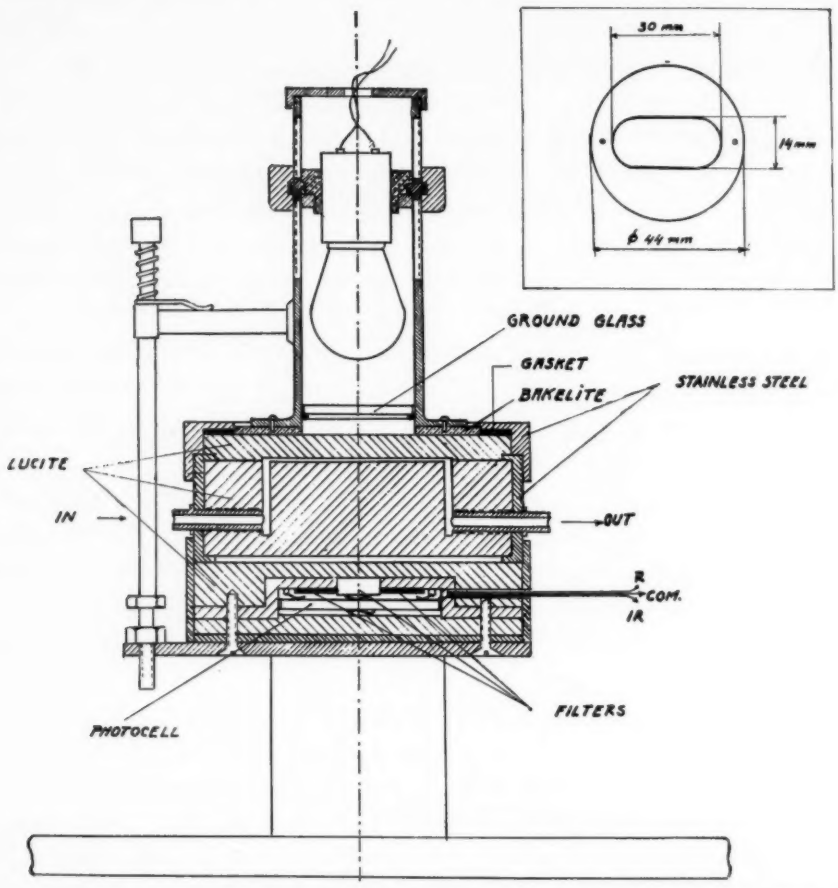


Fig. 3.—Cross-section view of the cuvette assembly. Volume capacity of the cuvette of 0.07 cm.; depth is 0.325 ml.

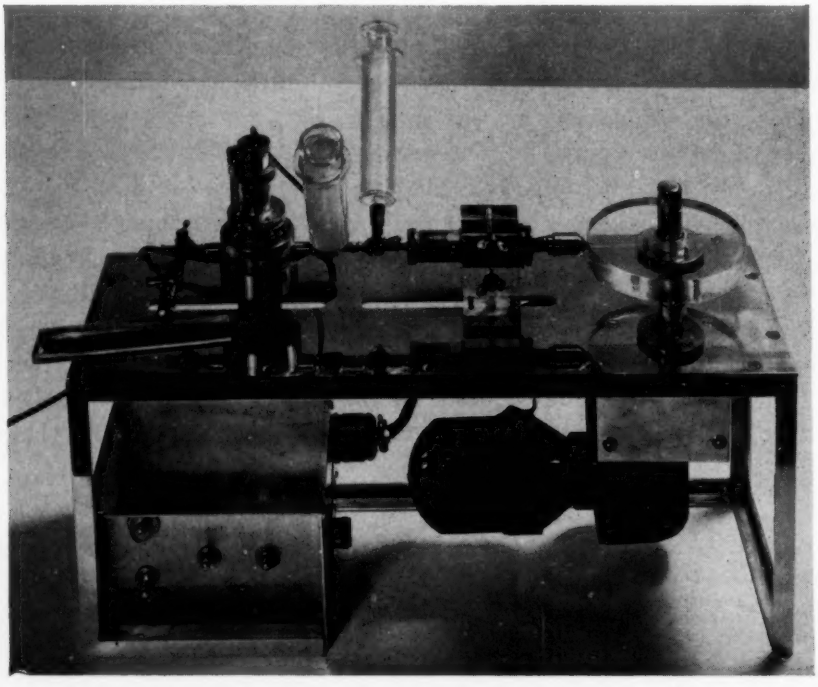


Fig. 4.—Photograph of the instrument showing the cuvette assembled, the drive to produce a constant rate of flow, and the accessories (oximeter not shown).

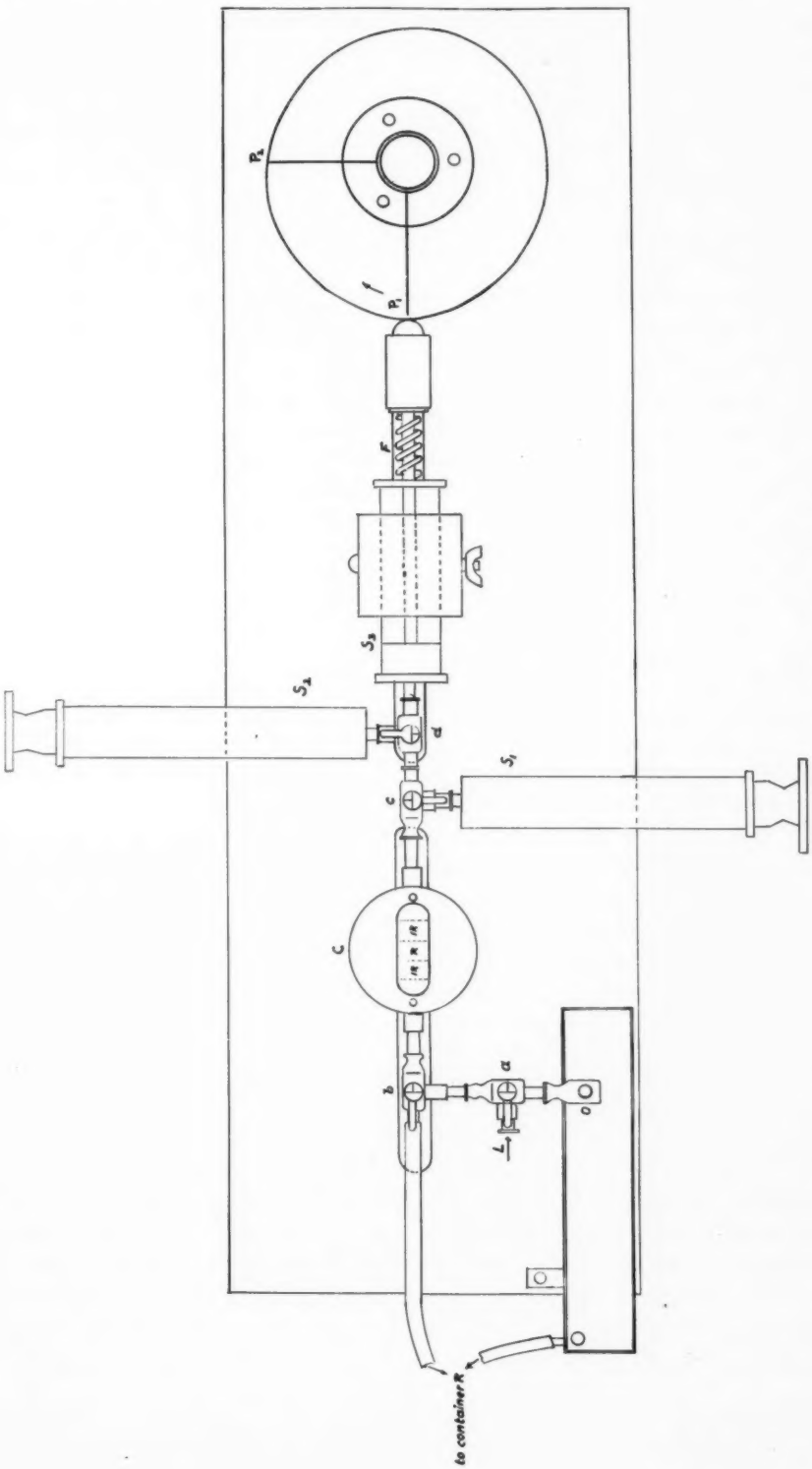


Fig. 5.—Functional drawing of the unit.

IR circuit. This resistance is preadjusted so that only one-half of the IR photocurrent flows when the operation selector switch is in position 3 (adjust) and saline is in the cuvette. Readings are taken with the selector switch in position 4 when blood is in the cuvette.

Fig. 3 shows the cross-section view of the cuvette assembly. The capacity of the cuvette of 0.07 cm. depth is 0.325 ml. The whole system from the orifice where the blood enters to the outflow orifice may be filled with less than 1 ml., but to operate the apparatus easily samples slightly in excess of this amount are required.

A photograph of the cuvette assembly and of the apparatus to provide a constant rate of flow is shown in Fig. 4, and a functional drawing of the unit can be seen in Fig. 5.

The filling is performed in the following manner: stopcocks *a* and *b* (type BD) are turned so that saline entering from line *L* via *a* may flow out via *b*, and a short length of rubber tubing into an empty container *R*. When it is ascertained that the inflow tube is free from air bubbles, the cuvette *C* is filled by drawing saline into syringe S_1 via stopcocks *b* and *c*. Stopcocks *c* and *d* are then turned so that the direction of flow points from syringe S_1 toward a 10 ml. calibrating syringe S_2, and the motor is started.* By means of an eccentric cam, *D*, fastened to the shaft of a reduction gear driven by the motor, and under the influence of an expanding spring *F*, saline is drawn from syringe S_1 into S_2. The rate and the constancy in the rate of flow, i.e., of the displacement of the piston of syringe S_2, is determined by the rate of rotation and contour of the cam. At the time the motor is started the spring is compressed and the end of the barrel is pressed against the side of the cam at point P_1. During the rotation of the cam the spring expands until point P_2 arrives in position facing the end of the barrel where it is compressed and saline is forced into syringe S_2 via stopcock *d*.

After the system has been filled with saline, the end of a cardiac catheter or a syringe with the blood may be placed into the orifice *O*. Stopcocks *a*, *b*, *c*, and *d* are turned so that the direction of flow points from *O* toward syringe S_3. The motor is started and the blood is drawn through the cuvette. As soon as point P_2 arrives in position facing the end of the barrel (or sooner if the quantity of the blood is less than 2 ml.), stopcock *d* is turned so that the blood is discarded into syringe S_2. Flushing of the cuvette is accomplished by forcing saline through the cuvette from syringe S_1 into the container *R* via stopcocks *c* and *b*; fresh saline is then drawn through the cuvette into syringe S_1 and the few drops of blood left in the tip at the entrance orifice are discarded. With a little experience, once the system had been carefully filled with saline, one determination, including readings on the double scale oximeter and flushing, may be accomplished in less than a minute.

CALIBRATION AND ACCURACY

The principles of bichromatic colorimetry, upon which the operation of the ear oximeter is based, have been employed in the construction of the oximeter for whole blood. The blood in the cuvette is transilluminated by the light of an incandescent bulb (6.0 volt, 0.5 amp.) operated at about 5.0 volts. The light transmitted through the layer of blood passes through two sets of optical filters for registration in two distinct spectral regions, the red and infrared, and then falls upon a barrier layer-type photocell, which is divided into three segments. The outputs of the infrared filtered photocells, consisting of the two outer segments connected in parallel, and that of the red filtered photocell, the mid-segment, are recorded simultaneously on a double beam galvanometer.

*The apparatus shown in Fig. 3 incorporates a 50 watt motor, the speed of which may be continuously varied between 1 and 3 r.p.m. by means of a friction-type reduction drive. In another unit, a 1/16 H.P. motor with Thyratron speed control is used. This latter drive has provided trouble-free operation during the past two years.

An empirical calibration diagram of the instrument is obtained by plotting the values of the percentage of oxygen saturation determined by the Van Slyke method against the ratios of the optical densities $\left(\log \frac{Dro}{Dr} \Big/ \log \frac{Dio}{Di}\right)$* obtained from the photoelectric readings in both spectral regions. In this expression Dro and Dr refer to deflections obtained with the red filtered photocell with saline and with blood in the cuvette, respectively. Dio and Di have similar meanings and refer to measurements with the infrared filtered photocell.† The operation of the whole blood oximeter developed by Wood[10] is based on a similar expression. Once the instrument is calibrated the values of the oxygen saturation of the unknown samples are read off the calibration curve. However, this procedure is laborious because calculations are involved, and logarithmic tables or slide rules have to be used or reference has to be made to rather complicated nomograms. In order to avoid these inconveniences a simple calculating device was constructed, which is similar to that described previously.[7] Provided the light intensity used for transillumination remains unchanged and the controls are set so that constant, predetermined galvanometer deflections are obtained with saline in the cuvette, Dro and Dio may be considered as apparatus constants and are entered as such in the construction of the calibrator. Only two readings, Dr and Di, are necessary to find the value of the oxygen saturation.

Fig. 6 shows the empirical calibration curve obtained when using 3 layers of Wratten No. 87 filter and 1 layer of Wratten No. 29 filter for the infrared and red bands, respectively. This diagram is based on 25 samples obtained in two subjects during cardiac catheterization and in three subjects by venous puncture. The venous samples were divided into two parts, one of which was oxygenated, then both parts were mixed in varying proportions. In this way samples of constant hemoglobin concentration and of different oxygen saturation were obtained. The manometric determinations and photoelectric measurements were carried out within a few hours after withdrawal. In the range from about 23 to 100 per cent oxygen saturation (the hemoglobin concentration varying from 9.2 to 15.7 Gm. per 100 ml.), the standard deviation of the differences was found to be 1.912 per cent oxygen saturation.

*The relationship which exists between the oxygen saturation and the ratio of the optical densities may be considered valid only for hemoglobin solutions and/or for whole hemolyzed blood (Kramer and associates loco citato). Because the total hemoglobin concentration of whole nonhemolyzed blood does not follow the classical laws of absorption, this relationship is considered only an approximation. It is of interest to note, however, that all the curves shown in Figs. 1 and 2 (and others recorded but not shown) appear to exhibit, in the range above approximately 5 Gm. per 100 milliliters, a linear relationship between the logarithm of the galvanometer deflection and the hemoglobin concentration.

†Note that when the cuvette is filled with saline, the red and infrared controls are adjusted in such a way that when the spot of the red galvanometer is brought to the zero position, Dio in millimeters on the galvanometer scale is equal to Dro in divisions on the dial scale. The oximeter circuit arrangement comprises a bias cell and the dial-scale serves as a very accurate means of measuring the bias current flowing in the circuit. Balance between the bias current and the output current of the red sensitive photocell is achieved when, by rotating the dial, the light spot of the upper (red) galvanometer is brought back into the zero position. The dial is provided with two scales: an upper linear and a lower logarithmic. The readings with the infrared photocell are taken with the lower galvanometer. The upper galvanometer scale is linear and the lower is logarithmic. (Experience has shown that the accuracy of the determinations is greatly increased with readings taken simultaneously on both upper and lower scales, because reading mistakes may be easily checked.) In two individual instruments used in this study the dial scale is divided into 100 divisions and 1 scale division corresponds to 4.55 mm. galvanometer deflection. The increased sensitivity of the estimations is due partly to the fact that the readings are not compressed into a narrow scale but are taken on an expanded scale with a "virtual" length of 455 mm.

It is of interest to note that the curve shown in Fig. 6 departs from linearity in the upper range of oxygen saturation. There is a close similarity between the deviation from linearity exhibited by this curve and the deviations exhibited by those obtained with ear oximeters using identical type of filter-photocell combinations. To investigate the effect of the band-width of the light used for transillumination on the evolution of the calibration curves, an

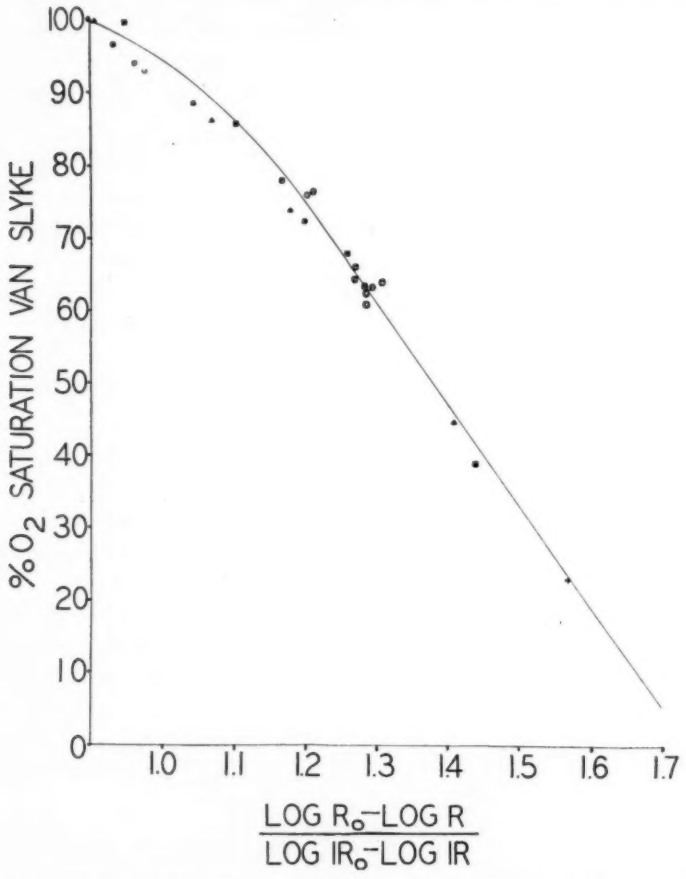


Fig. 6.—Empirical calibration diagram which resulted when 1 layer of Wratten No. 29 and 3 layers of Wratten No. 87 filters were used in the red and infrared regions, respectively. Note the marked deviation from linearity in the upper range of oxygen saturation.

interference filter (Schott-Jena, $\lambda max = 650$ mμ, Tmax = 54 per cent, and HW = 13 mμ) was substituted for the Wratten No. 29 previously used. The filters for the infrared light remained unchanged. Fig. 7 shows the straight-line relationship which resulted when this arrangement was used. This plot was constructed from manometric and photoelectric readings on 23 samples obtained by venous puncture in three subjects and by use of four different bottles of bank blood. Samples of constant hemoglobin concentration and of different oxygen saturation were prepared in the same manner as before. With the

oxygen saturation between 25 to 100 per cent and the hemoglobin concentration varying from 7.56 to 15.10 Gm. per 100 ml., the standard deviation of the differences was found to be equal to 1.790 per cent oxygen saturation.

In order to ascertain the extent to which the reproducibility and the variability in the estimations depend on instrumental and personal factors, the following experiment was devised. Determinations on the same samples were performed by two individuals using two instruments installed in two different laboratories. The photocells in the two instruments were carefully selected for similar spectral characteristics and interference filters were used

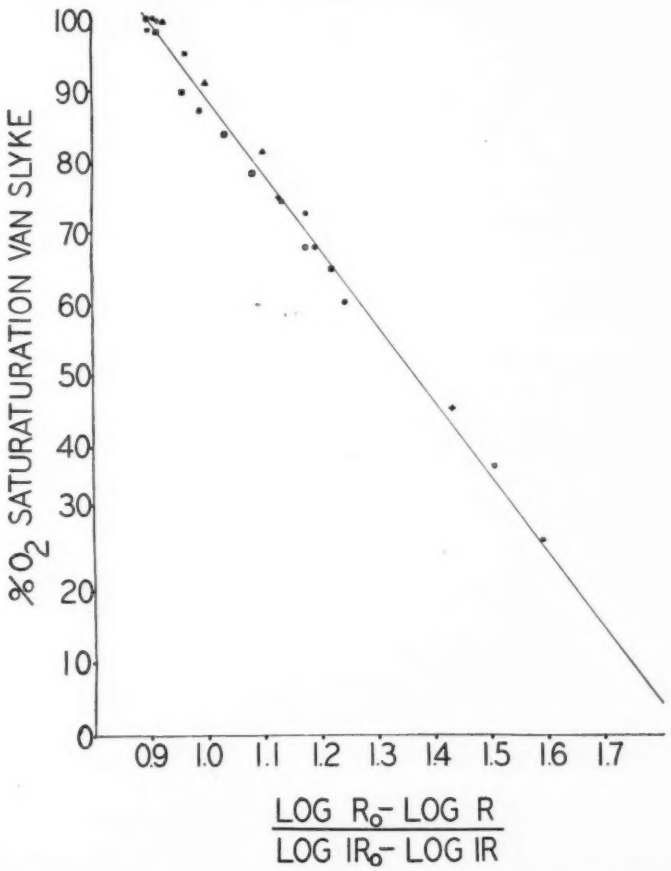


Fig. 7.—Empirical calibration diagram obtained when an interference filter (λmax = 650 mμ, and HW = 13 mμ) and 3 layers of Wratten No. 87 were used in the red and infrared regions, respectively.

in both. The rates of flow and the cuvettes were made similar. However, the distance between the light source and the layer of blood, and the light intensities were different, resulting in significantly different apparatus constants. One of the oximeters, already calibrated against manometric determinations, was operated by a skilled technician with some previous experience in its use, in the Cardiology Department of the Montreal Children's Hospital. The

determinations with the second instrument were carried out in the Department of Physiology, McGill University, by a fourth-year science student without any experience in its handling. Bank blood was used in these series of experiments. Samples with different oxygen saturation but of constant hemoglobin concentration were prepared by mixing in different proportions the oxygenated and unoxygenated parts of the blood of the same bottle. In order that samples of relatively high hemoglobin concentration might be produced, the blood of two bottles was centrifuged and a certain quantity of plasma removed prior to oxygenation and mixing. Soon after the manipulations on

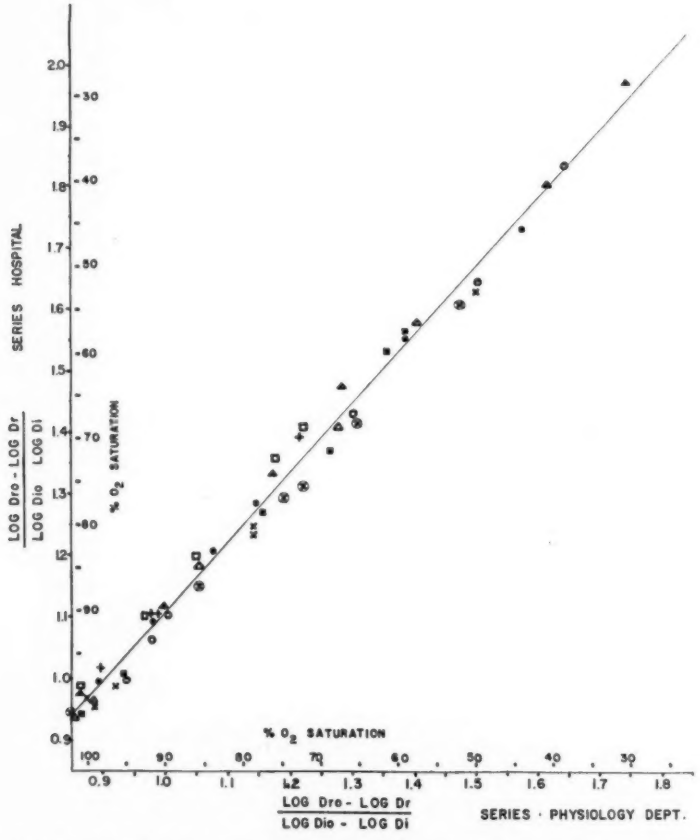

SERIES HOSPITAL
LOG Dro - LOG Dr / LOG Dio - LOG Di
% O2 SATURATION
% O2 SATURATION
LOG Dro - LOG Dr / LOG Dio - LOG Di
SERIES · PHYSIOLOGY DEPT.

Fig. 8.—Correlation between readings obtained with two similar instruments. The ratios of optical densities and the corresponding oxygen saturation values determined by the individual instruments are plotted against each other. The line of correlation does not pass through the origin. See text for explanation.

the samples were completed, the photoelectric readings were done in the hospital. The syringes, siliconed and heparinized in the usual manner and containing the remainder of the sample, were then capped with mercury and kept on ice until the readings started in the Physiology Department. The interval between readings with both instruments on the same samples varied somewhat, but they were all done on the same day. The results obtained in this series of experiments are illustrated in Fig. 8. In this diagram the ratios calculated

from readings with both instruments, on fifty samples, are plotted against each other. It will be noted that the line of correlation in this diagram does not pass through the origin. The reason for this, as already mentioned, is that the apparatus constants, hence the slopes of the calibration lines, were different for both instruments. Taking the oxygen saturation values obtained with the calibrated instrument as the basis for comparison, in the range from 28.3 to 100 per cent, the hemoglobin concentration varying from 9.3 to 17.8 Gm. per 100 ml., the standard deviation of differences was found to be 1.930 per cent oxygen saturation. The results obtained with both instruments are closely similar. The difference of the means was 0.22 per cent, and the correlation between the two sets of experiments was estimated to be $r = 0.995$. The standard deviation of differences between the photoelectrically determined oxygen saturation values and the values taken from the correlation line, for the individual instruments, were 1.365 and 1.370 per cent oxygen saturation. This implies that the photoelectric estimations can be reproduced with an accuracy within 2.74 per cent in 95 per cent of all the determinations.

Range and mean values of the oxygen saturations and of the hemoglobin concentrations, and the results obtained in this study, under different operating conditions, are given in Table I.

TABLE I

FILTER	PROCEDURE	NO. OF SAMPLES	HEMOGLOBIN (GM./100 ML.)	% O_2 SATURATION		S.D.D. (% O_2 SATURATION)	
				VAN SLYKE	CUVETTE		
Wratten No. 29 and 3 layers No. 87	Calibration	25 (5 subjects)	12.55	74.49	74.60	1.912	
			9.2 -15.7	22.8-100	23.2-100		
Interference and 3 layer Wratten No. 87	Calibration	23 (3 subjects and 3 bottles)	11.20	78.3	77.8	1.790	
			7.56-15.10	27.0-100	26.2-101.0		
	Comparison 2 cuvettes	50 (9 bottles)		Cuvette 1	Cuvette 2	Cuvette 1	Cuvette 2
			12.12	78.15	77.93	1.365	1.370
			9.3 -17.8	28.3-101.5	29.8-101.0		

CONCLUSIONS

The method described is both rapid and reliable for the in vitro estimation of oxygen saturation in small quantities of whole nonhemolyzed blood. The agreement between the results obtained by the Van Slyke and the photoelectric methods appeared to be satisfactory and adequate for most clinical applications. From the comparison of the results obtained by two cuvettes it is apparent that the technique used provides a high degree of reproducibility of the estimations over a wide range of oxygen saturation and erythrocyte concentration. These results may be interpreted as indicating that, because of the physical arrangement and circuitry used, the light scattering, which has been considered to be the main difficulty in performing satisfactory oxygen saturation estimations within the intact corpuscles, has been greatly reduced.

The accuracy of the determinations, about 85 per cent of which were made on samples having an oxygen saturation greater than 50 per cent, appears to be constant over the range of 50 to 100 per cent and independent of wide variation in the hemoglobin concentration. The accuracy of the estimations on a relatively small number of samples of low oxygen saturation was found to be of the same order as that obtained on samples in the higher range of oxygen saturation. As there was no progressive diminution of the apparent accuracy with the decrease in oxygen saturation, and since a linear relationship was observed between the logarithm of the photocurrent in the infrared and the hemoglobin concentration (above about 5 Gm. per 100 ml.), satisfactory performance might be expected in estimating the oxygen saturation over the whole range when the hemoglobin concentration exceeds 5 Gm. per 100 ml.

When employing the interference filter the precision appeared to be somewhat greater than that obtained when the gelatin filter was used. In addition to the increase in accuracy, the main advantage which results from utilization of a narrow band interference filter in place of a gelatin filter is that the calibration of the instrument becomes a relatively simple matter. Only a few calibrating samples and comparisons with the manometric method are required to construct the straight-line calibration diagram.

SUMMARY

A photoelectric method and apparatus for rapid estimation of the oxygen saturation within intact corpuscles are described.

Forty-eight comparisons were made with the Van Slyke method of analysis. Twenty-five of these were performed with an instrument employing the Wratten No. 29 gelatin filter and 23 with another using a narrow band interference filter.

A comparison between results obtained with two similar instruments on 50 samples of blood is reported.

In terms of the observed accuracy and reproducibility of the estimations the method appears to be adequate for routine clinical and laboratory applications.

We are indebted to Dr. A. S. V. Burgen for helpful discussion. The technical assistance of Mrs. C. Davis, B.Sc., and Mrs. J. Pollock, B.Sc., Department of Cardiology, Montreal Children's Hospital, and of Mr. C. Pinsky, B.Sc., and of Mr. G. Richard, Department of Physiology, McGill University, is acknowledged. The skill of Mr. Stanley Doig, Radiation Laboratory, McGill University, in the construction of the cuvette assemblies is greatly appreciated. Our thanks are due also to Mrs. J. Cloutte for secretarial assistance.

REFERENCES

1. Hall, F. G.: Spectroscopic Method for the Determination of Oxygen Saturation in Whole Blood, J. Biol. Chem. **130**: 573, 1939.
2. Nahas, G. G.: Spectrophotometric Determination of Hemoglobin and Oxyhemoglobin in Whole Hemolyzed Blood, Science **113**: 723, 1951.
3. Roos, A., and Rich, J. A.: Spectrophotometric Determination of Oxyhemoglobin Saturation and Oxygen Content of Blood, J. Lab. & Clin. Med. **40**: 431, 1952.
4. Wade, O. L., Bishop, J. M., Cumming, G., and Donald, K. W.: A Method for the Rapid Estimation of the Percentage Oxygen Saturation and Oxygen Content of Blood, Brit. M. J. **2**: 902, 1953.

5. Tsao, M. U., Sethna, S. S., Sloan, C. H., and Wyngarden, L. J.: Spectrophotometric Determination of the Oxygen Saturation of Whole Blood, J. Biol. Chem. **217**: 479, 1955.
6. Sekelj, P., Johnson, A. L., Hoff, H. E., and Pratt-Schuerch, M.: A Photoelectric Method for the Determination of Arterial Oxygen Saturation in Man, Am. Heart J. **42**: 826, 1951.
7. Sekelj, P.: Further Studies on Oximetry, Am. Heart J. **48**: 746, 1954.
8. Kramer, K., Elam, J. O., Saxton, G. A., and Elam, W. N.: Influence of Oxygen Saturation, Erythrocyte Concentration and Optical Depth upon the Red and Near-Infrared Light Transmittance of Whole Blood, Am. J. Physiol. **165**: 229, 1951.
9. Sendroy, J., Jr.: Manometric Determination of Hemoglobin by the Oxygen Capacity Method, J. Biol. Chem. **91**: 307, 1931.
10. Wood, E. H.: Oximetry, Otto Glasser's Medical Physics. **2**: 674, 1950.

ISOLATION OF SERUM LIPOPROTEINS BY ZONE ELECTROPHORESIS

JOSEPH BERNSOHN, PH.D.
HINES, ILL.

THE isolation of α- and β-lipoproteins from serum for further study usually is accomplished by complex techniques such as ultracentrifugation[1] and alcohol fractionation.[2] Separation of proteins by electrophoresis on paper has been employed for quantitative work, but apart from the staining difficulties usually encountered, the method is of limited value because of the small amounts of material used. This objection can be overcome by zone electrophoresis techniques using paper or starch as supporting media. But the isolation of the protein is on an empirical basis, and since the block usually is sectioned into small segments for analytic purposes[3] no isolation of discrete protein fractions occurs, and only small amounts of material are available in each segment for analysis. McDonald and Bermes[4] recently have reported a method for staining of the serum with acetylated sudan black B prior to separating the proteins by ionography. They state that the dye is in physical solution in the lipid material of the serum and migrates with the two major lipoprotein bands. If such a technique could be applied to zone electrophoresis, the α- and β-lipoprotein fractions could be visualized and thus separated in toto from the remaining serum protein fractions.

METHOD

The apparatus as described by Ackermann and associates,[5] with some modifications, was used. In place of the cellulose sponges, the starch block was prepared on Whatman No. 3MM filter paper, which lined the bottom of the tray and whose ends dipped into the buffer vessels.[6] Acetylated sudan black B was added to the serum and the procedure as described by McDonald and Bermes was followed rigidly. Two milliliters of the serum was placed in a groove in the starch by means of a syringe with a 20-gauge needle and the starch replaced by pushing it in from the ridges on each side of the groove. A current of 25 to 30 Ma. at 500 to 600 volts was applied, and the run was terminated at the end of 16 to 20 hours. Barbital buffer, pH 8.6, ionic strength 0.05, was used throughout.

RESULTS

In Fig. 1, a photograph of the starch block after the separation is presented. Three bands are visible: (1) the origin containing dye which has not migrated, (2) the dark β-lipoprotein band which is near the origin, and (3) the more rapidly moving α-lipoprotein band. Some tailing of the two

From the Neuropsychiatric Research Laboratory, Veterans Administration Hospital, Hines, Ill., and Northwestern University Medical School, Chicago, Ill.

This work was aided by a grant from the Multiple Sclerosis Foundation, Lewis J. Pollock, M.D., principal investigator.

Received for publication July 23, 1956.

lipoproteins also occurs but these are minor components comprising about 10 to 15 per cent of the total dye that has migrated. The two blue-dyed bands can now be cut out of the starch block, and the dye, protein, and lipid material can be extracted.

It appears that the dye is now water-soluble and insoluble in the usual lipid solvents. Since the dye is originally insoluble in water, this finding was unexpected. Spectrophotometric analysis of the dye from 240 to 700 mμ, which was eluted from the starch with water, showed absorption characteristics similar to the original alcohol-soluble dye. When the dyed serum was dialyzed against distilled water, the dye did not migrate through the membrane. Thus it would seem that the dye is linked to the lipoprotein molecule in chemical combination rather than in simple physical solution, and now possesses solubility properties similar to the protein moiety.

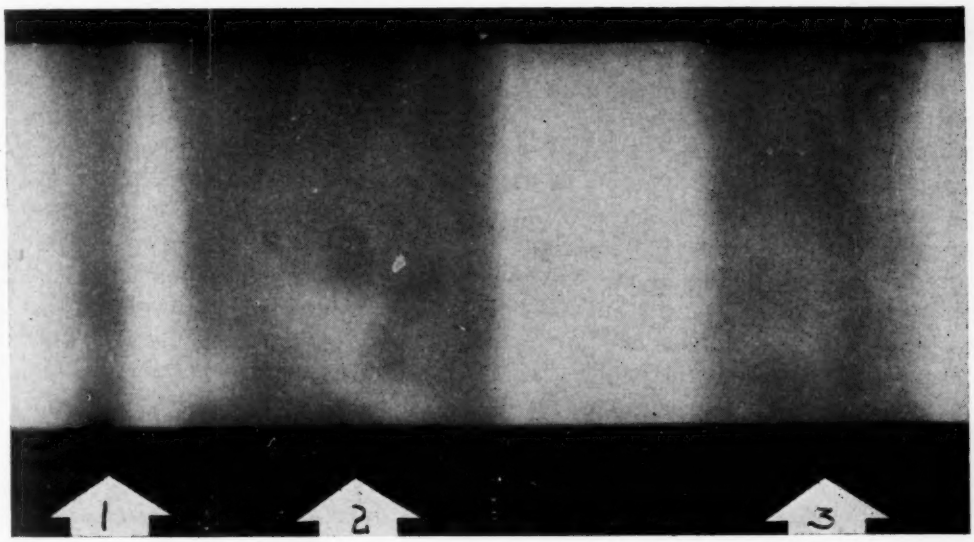


Fig. 1.—Appearance of starch block after separation of acetylated sudan black B dyed serum. Arrows from left to right mark (*1*) origin, (*2*) β-lipoprotein, and (*3*) α-lipoprotein.

In order that it might be ascertained that the position of the two stained bands corresponds to the α- and β-lipoproteins, a starch preparation was run simultaneously with the same serum sample, not dyed, and a paper print of the pattern was stained with bromphenol blue. When this strip was placed side by side with the pattern obtained by the sudan black B dyed serum, it could be seen that the β-lipoprotein band migrated just behind the β-globulin fraction, and the α-lipoprotein band lay between the albumin and α-globulin with some overlapping of the albumin fraction. These relationships were similar to those described by Kunkel and Slater.[3]

Extraction of the dye and protein was carried out in 40 ml. round-bottom centrifuge tubes. The starch was extracted five times with distilled water and decanted after each extraction. The decantates were made up to volume. Dye intensity was determined directly at 620 mμ and protein was measured by the

Folin-Ciocalteu reagent. The average of six normal subjects showed that 30.2 per cent of the migrating dye was bound in the α-lipoprotein fraction and 69.8 per cent in the β-lipoprotein fraction. Recovery of protein from the starch block averaged 81.7 per cent of the total protein applied at the initial boundary.

REFERENCES

1. Gofman, J. W., Lindgren, F. T., and Elliott, H.: Ultracentrifugal Studies of Lipoproteins of Human Serum, J. Biol. Chem. **179**: 973-979, 1949.
2. Russ, E. M., Eder, H. A., and Barr, D. P.: Protein-Lipid Relationships in Human Plasma, Am. J. Med. **11**: 468-479, 1951.
3. Kunkel, H. G., and Slater, R. J.: Lipoprotein Patterns of Serum Obtained by Zone Electrophoresis, J. Clin. Invest. **31**: 677-684, 1952.
4. McDonald, H. J., and Bermes, E. W., Jr.: A New Procedure for Staining Lipoproteins in Ionographic Separations, Biochim. et Biophys. Acta. **17**: 290, 1955.
5. Ackermann, P. G., Toro, G., and Kountz, W. B.: Zone Electrophoresis in the Study of Serum Lipoproteins. I. Methods and Preliminary Results, J. LAB. & CLIN. MED. **44**: 517-530, 1954.
6. Kinnory, D. W.: Unpublished data.

A MICROMETHOD FOR THE DETERMINATION OF SALICYLIC ACID IN PLASMA

SING-PAO CHIANG, PH.D., AND SMITH FREEMAN, M.D., PH.D.
CHICAGO, ILL.

INTRODUCTION

THE most generally used method for the determination of salicylates in plasma is that developed by Brodie, Udenfriend, and Coburn.[1] The estimation depends upon the reaction of ferric ion and phenol, which produces a reddish-violet color. Before the application of this colorimetric reaction, however, the salicylates must be extracted from the acidified plasma with an organic solvent and then returned to the aqueous phase for color development. This involves several steps of manipulation that are time consuming and possible sources of error. The volume of plasma necessary to make a determination, 1 to 2 ml., appears to be quite large as applied to small children or in connection with other studies requiring a large volume of plasma.

The present paper describes a micromethod developed with the purpose of eliminating the two extractions involved and also of employing a small quantity of plasma. The procedure consists of a direct separation of the salicylic acid from plasma by paper chromatography with ethanol as a resolving solvent and a colorimetric estimation of the salicylic acid extracted from the paper with water and ferric ion. The determination can be carried out on 20 μl of plasma and is sensitive to 0.5 mcg.

EXPERIMENTAL

I. *Reagents and Materials.*—

1. Absolute ethanol
2. Hydrochloric acid 6 N
3. Ferric nitrate 1 per cent in 0.07 N HNO_3
   (13.4 ml. of concentrated HNO_3 dilute to 1 liter)
4. Constricted type micro-pipettes (Linderstrom-Lang-Holter)*
5. Paper strips—3 MM Whatman filter paper ruled and cut as shown in Fig. 1. The strips should be extracted in Soxhlet extractor with ethanol for 8 hours and air-dried before they can be used.
6. *Standard solutions*: Stock solution salicylic acid: 1 mg. per milliliter. If kept in the refrigerator, it is stable for months.
   Working standards: 100 mcg. per milliliter = 1-10 of stock
   200 mcg. per milliliter = 1-5 of stock
   400 mcg. per milliliter = 2-5 of stock
7. Beckman spectrophotometer with micro attachments.†

From the Department of Biochemistry, Northwestern University Medical School, Chicago, Ill.

Received for publication July 25, 1956.

*Microchemical Specialties Co.

†Pyrocell Company.

II. *Procedure.*—1. *Application of sample to the paper strip with use of micro-pipette:* Twenty microliters of plasma is applied to the paper strip at the designated line (Fig. 1). Allow to almost dry in air at room temperature. Acidify the plasma by adding 20 μl of 6 N HCl onto the same spot as for the plasma. The acid should wet the entire area covered by the plasma. Allow to air dry.

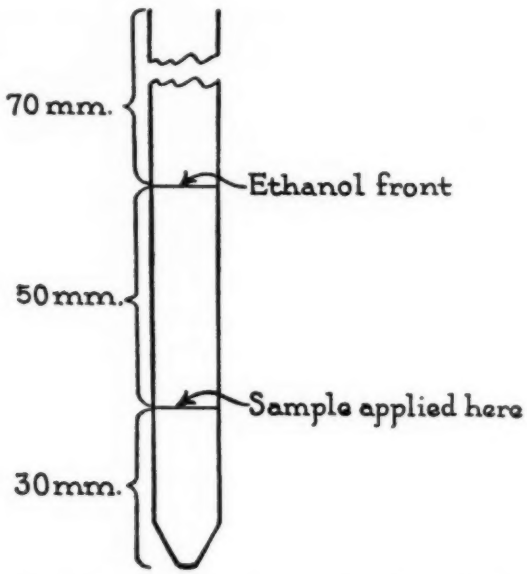


Fig. 1.—Separation of salicylic acid from plasma by paper chromatography.

2. *Paper chromatography:* Ascending paper chromatography is carried out by the placing of the paper strips in test tubes 150 × 19 mm. containing 1 ml. of ethanol. Since salicylic acid is very soluble in ethanol, it ascends the strip with the ethanol front. When the solvent front reaches the intended line, the paper strip is taken out of the test tube and allowed to dry in the air. Repeat this procedure three more times.

3. *Colorimetric estimation:* Cut from the paper strip an area 3 mm. above and 7 mm. below the line representing the solvent front. All of the salicylates should be on this piece, which is folded and put into a small test tube (2 ml.). Five hundred microliters of distilled H_2O and 20 μl of $Fe(NO_3)_3$ reagent are added. Mix well and allow thirty minutes for extraction and development of color with frequent thorough mixing. Read the optical density in a Beckman spectrophotometer at 540 mμ in micro cells.

4. *Standards:* For standards, 20 μl of salicylate-free plasma is dried on a number of paper strips. They are acidified and dried again as for the samples. Then 20 μl of standard solvents containing 100, 200, and 400 mcg. per milliliter are added to the spots covered by the plasma. After drying, follow exactly the same procedure as that for the samples.

III. *Calculation.*—

1. *Average Kμg of salicylic acid from standards:*

Example:

SALICYLIC ACID	O.D. × 10^3	Kμg × 10^3
2 mcg.	40	20
	42	21
4 mcg.	80	20
	82	20
8 mcg.	152	19
	160	20

Average Kμg = 0.020.

2. *Plasma levels:* Let a = O.D. of the patient's plasma at 0 hour.
A = O.D. of the same patient's plasma at some particular interval after ingestion of salicylates.

$$\text{Then } \frac{A - a}{\text{Av. K}\mu g} \times \frac{100}{20^*} = \text{mcg./100 } \mu\text{l or mg./100 ml.}$$

Example: a = 0.010 A = 0.123

$$\frac{0.123 - 0.010}{0.020} \times \frac{100}{20} = 28 \text{ mg./100 ml. or 28 mg. per cent.}$$

RESULTS

Fig. 2 shows the standard curve obtained by this method as applied to standard solutions of salicylic acid. Linear function is clearly demonstrated over the range studied.

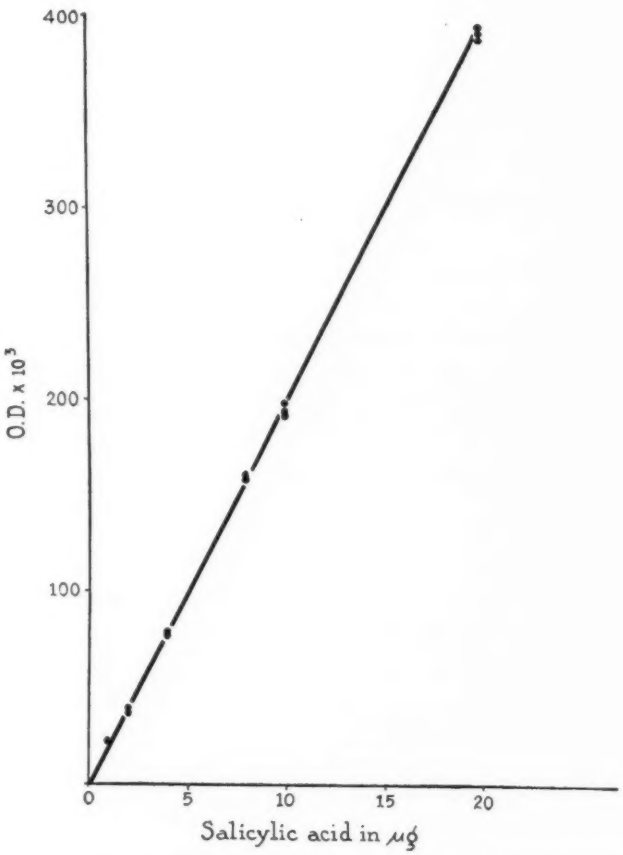


Fig. 2.—Standard curve for salicylic acid determination.

The results of recovery experiments are assembled in Table I. The amount recovered varies from 85 to 115 per cent, averaging 96 per cent. The values are reproducible from plasma samples that have been kept in the refrigerator for two weeks or longer.

*Volume of pipette.

TABLE I. SHOWING THE RECOVERY OF SALICYLIC ACID ADDED TO PLASMA

PLASMA NO.	SALICYLIC ACID IN MCG. IN PLASMA	ADDED TO PLASMA	FOUND	RECOVERED	% RECOVERY
3	5.6	2	7.5	1.9	95
		2	7.4	1.8	90
		4	9.1	3.5	88
		4	9.2	3.6	90
		8	13.3	7.7	96
		8	13.7	8.1	101
4	7.0	2	8.7	1.7	85
		2	9.3	2.3	115
		4	10.7	3.7	93
		4	10.7	3.7	93
		8	15.2	8.2	102
		8	14.5	7.5	94
9	4.6	2	6.4	1.8	90
		2	6.5	1.9	95
		4	8.4	3.8	95
		4	8.2	3.6	90
		8	12.3	7.7	96
		8	12.7	8.1	101
10	3.7	2	5.6	1.9	95
		2	5.8	2.1	105
		4	7.5	3.8	95
		4	7.8	4.1	102
		8	12.1	8.4	105
		8	11.9	8.2	102

TABLE II. PLASMA SALICYLATE LEVELS ON PATIENTS RECEIVING ACETYL SALICYLIC ACID

PATIENT	TIME IN HR. AFTER SALICYLATE INGESTION	O.D. $\times 10^3$	SALICYLATE IN MG. %
A	0	12	0
		14	0
	4	100	25
		100	25
	8	118	29
		123	31
B	0	9	0
		10	0
	4	88	22
		89	22
	8	109	28
		105	27
C	0	14	0
		10	0
	4	100	25
		100	25
	8	130	33
		130	33
D	0	13	0
		11	0
	4	66	15
		66	15
	8	126	32
		127	32

Table II gives the results obtained on samples of plasma from patients before and 4 and 8 hours after receiving 90 mg. per kilogram of acetyl salicylate orally over a four-hour period.

DISCUSSION

The above method is simplified from Brodie's[1] by the use of paper chromatography. The separation of salicylic acid from plasma protein is definite and complete as evidenced by the failure to produce the violet color on the remaining part of the paper strip when it was sprayed with a dilute solution of the ferric nitrate reagent. Ethanol was chosen as a resolving agent because it is one of the best solvents for salicylic acid, and also it moves at a suitable rate for simple ascending paper chromatography. Other solvents, such as chloroform, ethyl ether, methylene dichloride, and petroleum ether, have been tried. They either moved too fast to carry salicylic acid along or did not dissolve salicylic acid as readily as ethanol.

The acidity of the final solution in which the color is to be produced is of extreme importance. The intensity of the color varies with the acidity and is reproducible only within a pH range of 3.0 to 5.0. Therefore, it is important to treat the standards as previously described.

The results obtained from the patient's plasma samples are comparable to the values as reported by Smith and associates.[2]

The method is very simple. One can easily carry out 20 to 30 determinations during a workday. Because of the small volume of plasma required, small experimental animals may be used for the study of salicylate metabolism. Several samples of dog plasma have been analyzed by this method and no difficulty has been encountered. By the use of larger volumes of plasma, other spectrophotometers or colorimeters with cells of 1 to 2 ml. capacies may be used.

Preliminary observations indicate that this procedure may be applied to the estimation of free salicylic acid in urine.

SUMMARY

A convenient accurate micromethod for the separation of salicylates from plasma protein is described and applied to the colorimetric estimation of salicylates in plasma.

REFERENCES

1. Brodie, B. B., Udenfriend, S., and Coburn, A. F.: The Determination of Salicylic Acid in Plasma, J. Pharmacol. & Exper. Therap. **80**: 114-117, 1944.
2. Smith, P. K., Gleason, H. L., Stoll, C. G., and Ogorzalek, S.: Studies on the Pharmacology of Salicylates, J. Pharmacol. & Exper. Therap. **87**: 237-255, 1946.

A TITRATION METHOD FOR THE DETERMINATION OF CALCIUM IN SERUM USING A NEW INDICATOR

MARIE A. ANDERSCH, PH.D.
BALTIMORE, MD.

SEVERAL procedures for the determination of calcium in serum based on the titration of this ion with a chelating agent have been reported.[1-5] These methods have not gained wide use, however, because the indicators used have not been entirely satisfactory. Ammonium purpurate has a poor visual end point and Ehrichrome Black T is not specific for calcium in the presence of magnesium. Recently a new indicator, specific for calcium in the presence of magnesium, has been used in water analysis.[6] At a highly alkaline pH the color change, from yellow-green in the presence of the calcium ion to brown in its absence, is relatively easy to detect.

We therefore have investigated the use of this indicator in a method for the determination of calcium based on the chelation of this ion with ethylenediaminetetraacetic acid. The titration was carried out on an aliquot of a trichloracetic acid filtrate adjusted to a pH above 12.5. The method yielded values which did not differ statistically from those obtained by the Clark-Collip[7] modification of the Kramer-Tisdall method[8] when either the equivalent of 0.2 ml. or of 1.0 ml. of serum was used.

MATERIAL AND REAGENTS

1. A 2 ml. microburette graduated in 0.01 ml.

2. Ten per cent trichloracetic acid.

3. Ten per cent sodium hydroxide. The addition of 1 Gm. of sodium cyanide per 100 ml. sharpens the end point somewhat.

4. Standard calcium solution.

Stock: Dry the reagent grade of calcium carbonate at 105° C. for 24 hours. Weigh out 2.5 Gm. and transfer to 1 L. volumetric flask. Add about 200 ml. of water and 50 ml. of 1 N HCl in order to convert the carbonate to the chloride. Dilute to volume. Calcium = 100 mg. per 100 ml.

Working standard: Transfer 10 ml. of the stock standard to a 100 ml. volumetric flask and dilute to volume. Ca = 10 mg. per 100 ml.

5. Calcein* indicator. Dissolve 2 Gm. of the Calcein in 25 ml. of 1 N sodium hydroxide and dilute to 250 ml. with water. The indicator is stable at room temperatures.

6. Disodium dihydrogen ethylenediaminetetraacetic acid (EDTA).

Solution No. 1: Dissolve 0.93 Gm. of the salt in water and make up to 1 liter.

Solution No. 2: Transfer 20 ml. of solution No. 1 to a 100 ml. volumetric flask and dilute to volume.

From the Division of Clinical Pathology, Department of Medicine of the University of Maryland Medical School, Baltimore, Md.

Received for publication July 28, 1956.

*Available from the G. Frederick Smith Chemical Co., Columbus, Ohio.

7. Standardization of EDTA. Measure 1.0 ml. of the calcium working standard into a 50 ml. Erlenmeyer flask. Add 4 ml. of 10 per cent trichloracetic acid, 1.5 ml. of 10 per cent sodium hydroxide, and 1 drop of indicator. Titrate with EDTA No. 1 as outlined under procedure. Blank: Substitute 1 ml. of water for the standard and proceed as directed above.

The titration of the standard minus the titration of the blank represents the EDTA necessary for the chelation of 0.1 mg. calcium. The EDTA may be adjusted so that 1.0 ml. is required for the titration.

METHOD

Macrotitration.—Transfer 8 ml. of 10 per cent trichloracetic acid to a small flask and add 2 ml. of serum with shaking. Smaller volumes of serum may be precipitated with use of the same proportions. Filter the mixture through an ashless filter paper or transfer the material to a tube, centrifuge for a few minutes, and then filter. A portion of the filtrate may be used for the determination of serum phosphorus.

Transfer 5 ml. of the filtrate to a 50 ml. Erlenmeyer flask and add 1.5 ml. of 10 per cent sodium hydroxide and one drop of indicator.

Titrate with the EDTA solution No. 1. The flask should be shaken vigorously during the titration, as calcium hydroxide tends to precipitate in the highly alkaline solution. The color change from yellow-green to brown is seen best with a low intensity, non-fluorescent illumination.

Blank: Substitute water for serum in the procedure.

Standard: Substitute standard for serum in the procedure. It is not necessary to run a standard each time. The EDTA is stable in solution.

Calculation:

B = titration of the blank in milliliters.
S = titration of the standard in milliliters.
U = titration of the unknown in milliliters.

$$\frac{U-B}{S-B} \times 0.1 \times 100 \text{ mg.} = \text{calcium per 100 ml.}$$

Semimicro Titration.—Smaller quantities of filtrate may be titrated. The agreement with the standard method is not significantly altered when as little as 1 ml. of the filtrate is used.

Proportionately smaller quantities of sodium hydroxide should be added and the EDTA solution No. 2 used for the titration.

Blank and standard: Prepare as previously outlined under the macrotitration and titrate quantities equivalent to the amount of the filtrate used for the determination of the calcium in the unknown.

Calculation:

F = ml. of filtrate titrated.

$$\frac{U-B}{S-B} \times 0.02 \times \frac{5}{F} = \text{mg. calcium per 100 ml.}$$

RESULTS AND DISCUSSION

The calcium in the serum of 25 patients was determined by the Clark-Collip method and by the macrotitration procedure. The calcium was also determined in 10 of the same sera with use of only 1 ml. portions of the filtrate. Although duplicate determinations were usually run, in a few instances there was only enough serum for a single determination by the reference method. The results are tabulated in Tables I and II.

TABLE I. A COMPARISON OF THE CALCIUM VALUES OBTAINED BY TITRATION WITH THOSE OBTAINED BY A STANDARD METHOD

SAMPLE NO.	TITRATION		CLARK-COLLIP
	MACRO	SEMIMICRO	
1.	10.2	10.1	10.2
2.	9.0	9.3	9.1
3.	8.3	8.3	8.7
4.	7.8	7.9	7.9
5.	9.8	9.5	9.6
6.	5.9	5.8	5.8
7.	9.5	9.7	9.6
8.	9.6	9.5	9.8
9.	13.6	14.0	13.8
10.	10.5	9.7	10.2
11.	9.0		9.1
12.	9.2		9.4
13.	9.4		9.6
14.	14.2		14.0
15.	9.4		9.0
16.	4.6		4.7
17.	11.1		11.5
18.	9.2		9.5
19.	8.7		9.0
20.	10.5		10.2
21.	10.0		10.1
22.	10.5		10.1
23.	8.9		8.6
24.	8.9		8.6
25.	10.1		10.0

All values are expressed in milligram per 100 milliliter of serum.

TABLE II. A STATISTICAL COMPARISON OF THE VALUES FOR CALCIUM DETERMINED BY THE CLARK-COLLIP METHOD AND BY THE MACRO- AND SEMIMICROTITRATION METHODS

	NO. SERA	MEAN OF DIFFERENCE	S.D. OF MEAN	S.E. OF MEAN
Clark-Collip vs. macro	25	−0.04	±0.24	0.05
Clark-Collip vs. semimicro	10	−0.06	±0.30	0.10

Values are expressed in milligrams.

The agreement between the results obtained by the reference method and the macro- and semimicrotitrations was always within 5 per cent. The "t" values calculated from the paired data indicated that the differences between the values obtained by the macrotitration and the reference method as well as those obtained by the semimicrotitration and the reference methods did not vary significantly from zero.

Since calcium and phosphorus may be determined on 2 ml. of serum, the macrotitration should be satisfactory for routine use, while the semimicro procedure would give satisfactory values when only limited quantities of serum are available for the determination of calcium and phosphorus.

SUMMARY

1. A new indicator, specific for calcium in the presence of magnesium, has been used for the determination of serum calcium in a procedure based on the titration of serum calcium with ethylenediaminetetraacetic acid.

2. The results are not statistically different from those obtained by the Clark-Collip method when either the equivalent of 1 ml. or of 0.2 ml. of serum is titrated.

REFERENCES

1. Buckley, E. S., Jr., Gibson, J. B., II, and Bartolette, T. R.: Simplified Titrimetric Technique for the Assay of Calcium and Magnesium in Plasma. J. LAB. & CLIN. MED. **38**: 751, 1951.
2. Elliott, W. E.: Volumetric Determination of Calcium in Blood Serum, J. Biol. Chem. **197**: 641, 1952.
3. Kilbrick, F. C., Ross, M., and Rogers, H. E.: Microdetermination of Calcium in Blood Serum by Direct Titration, Proc. Soc. Exper. Biol. & Med. **81**: 351, 1952.
4. Fales, F. W.: A Microdetermination for the Determination of Calcium, J. Biol. Chem. **204**: 577, 1953.
5. Sobel, A. E., and Hanok, A.: A Rapid Method for the Determination of Ultramicro Quantities of Calcium and Magnesium, Proc. Soc. Exper. Biol. & Med. **77**: 737, 1951.
6. Diehl, H., and Ellingboe, J. L.: Indicator for the Titration of Calcium In Presence of Magnesium, Anal. Chem. **28**: 882, 1956.
7. Clark, E. P., and Collip, J. B.: A Study of the Tisdall Method for the Determination of Blood Serum Calcium With a Suggested Modification, J. Biol. Chem. **63**: 461, 1925.
8. Kramer, B., and Tisdall, F. F.: A Simple Technique for the Determination of Calcium and Magnesium in Small Amounts of Serum, J. Biol. Chem. **47**: 475, 1921.

A METHOD FOR CONCENTRATING AND DETERMINING IODIDE IN URINE

HAROLD L. HELWIG, PH.D., WILLIAM A. REILLY, M.D., AND
JAMES N. CASTLE, A.B.
SAN FRANCISCO, CALIF.

THE determination of iodide ion in urine is usually limited by the use of micromethods which require rigid control of laboratory environment to reduce contamination to a minimum. The use of a less sensitive analytic measuring method for urinary iodide requires a technique for concentrating this halide, usually by laborious evaporation and ashing of urine samples. Urinary iodide concentrations are very useful values for calculating urinary iodide specific activities and plasma iodide specific activities. These values are useful, in turn, in connection with studies of iodine and thyroid gland metabolism. In an effort to make this information easily available, we developed the following method for concentrating and analyzing urinary iodide.

APPARATUS

Columns.—One centimeter bore glass tubing 10 cm. in length, with a short tip having a 1 mm. bore, is used to contain a AgCl-cellulose column. The column is prepared by adding in succession from the tip upward the following: a 1 cm. layer of glass wool; a 0.5 cm. layer of cellulose powder suspended in 0.1 N H_2SO_4; a layer of AgCl-cellulose powder, as prepared under "Reagents;" a 0.5 cm. layer of cellulose powder; and a 1 cm. layer of glass wool.

Spectrophotometer.—A Beckman spectrophotometer Model D U with 1 cm. Corex cuvettes was used.

Counter.—A Tracerlab Superscaler and NaI (Tl) crystal, well or flat type, were used to assay I^{131} activity.

REAGENTS

AgCl Column.—To 1 Gm. of B quality Whatman cellulose powder in 100 ml. distilled water is added 0.1 Gm. $AgNO_3$, analytic grade. The suspension is acidified with sulfuric acid by adding 0.05 ml. concentrated H_2SO_4 per 100 ml. suspension. A slight excess of NaCl, A.C.S. grade, is added with stirring. Thirty-three milliliters of this suspension is used in preparing the AgCl-cellulose column. The column is then washed with 100 ml. of 0.1 N H_2SO_4 to remove excess chloride and any free AgCl colloid.

Silver Nitrate.—A.C.S. grade (Merck).

Sodium Chloride.—A.C.S. grade, 0.002 per cent iodide (Merck).

Potassium Permanganate C.P.—Saturated aqueous solution (Fisher-Scientific).

Sodium Nitrite.—Ten per cent aqueous solution (City Chemical Company).

Urea.—C.P. grade (Dupont).

From the Radioisotope Service, Veterans Administration Hospital, San Francisco, Calif.
Received for publication July 31, 1956.

Bromine.—A.C.S. grade, 0.05 per cent iodine (J. T. Baker). This reagent is distilled under copper sulfate solution to remove iodine. Five-hundredths normal H_2SO_4 is saturated with the distilled bromine.

CONCENTRATION OF URINARY IODIDE

The modified method of Purves[1] for concentrating urinary iodide is used. If protein is present in the urine, its removal facilitates filtration through the AgCl-cellulose column. Care should be taken to keep urinary iodide in the reduced state by minimizing exposure of specimens to light and by storing them in cold temperature and in light-tight containers. Formation of colloidal AgCl is avoided by acidifying all solutions coming in contact with the column.

Procedure.—1. To 200 ml. of urine (50 to 2,000 ml. can be used) add carrier-free I^{131} that has been reduced with Na_2SO_3 to give such a counting rate that 10 ml. will contain enough counts per second per ml. (c/s/ml.) at the time of the final step of the analysis to be accurately assayed. Five hundred counts per second per ml. are adequate.

2. Accurately determine the actual c/s/ml. of urine.

3. Acidify the 200 ml. of urine to pH 5 with sulfuric acid and heat rapidly to 95° C.; filter to remove protein, cool, and while stirring, add 0.5 ml. concentrated H_2SO_4 until the urine is 0.1 N with acid. Pour the cooled urine into a funnel attached with rubber tubing to the AgCl-cellulose column. Discard the effluent which will contain less than 10 per cent of the iodide radioactivity. Wash the column with 200 ml. of 0.1 N H_2SO_4, and discard washings. To remove the iodide from the column, add 15 ml. of Br_2-saturated 0.05 N H_2SO_4 and collect the eluate containing the iodine which now is iodate.

DETERMINATION OF IODIDE

The urinary iodide, concentrated in a volume of 15 ml. as iodate ion, is freed of reducing substances and then measured in a spectrophotometer. Reducing substances are partially removed by thorough acid washing of the column after passage of the urine through the column, the procedure described in the previous step. Overheating is avoided during the acid washing to prevent loss of iodate; the critical temperature is about 80° C. Also to be avoided is a reduction of volume of the acid solution of iodate at reduced temperatures, for this causes increase of acid concentration and in turn loss of iodate ion.

Procedure.—Heat the eluate from the column containing Br_2, $I^{131}\overset{127}{O_3^-}$, and unknown substances from urine, in a 50 ml. beaker at 70° C. in a water bath for an hour to remove Br_2. Continue heating and add 1 drop of saturated aqueous $KMnO_4$ to oxidize any further unknown reducing substances that may be present. After ten minutes, add 1 to 2 drops of 10 per cent $NaNO_2$ to destroy oxides of manganese. After 10 minutes of heating add solid urea to destroy traces of HNO_2. During the preceding steps water should be added to prevent decrease of the volume. Adjust the pH to 2.8 with $NaHCO_3$ and add 1 ml. of citrate-phosphate buffer of pH 2.8 (16.83 ml. 0.1 N citric acid + 3.17 ml. 0.2 M Na_2HPO_4). Place an aliquot of resulting solution in a Corex cuvette into the Beckman spectrophotometer and adjust the instrument to zero optical density at 352 mμ. Add a drop of 50 per cent aqueous KI or NaI and mix by inversion. Return cuvette to the instrument and measure the optical density of the iodine released. Determine the concentration of stable iodine from a standard curve of optical density versus concentration of iodine as iodate, covering the range shown in Table II.

Pipette an aliquot, e.g., 1 ml. of the reaction mixture, in the cuvette and measure its radioactivity. Calculation:

$$\text{Urinary I- } (\mu g/ml.) = \frac{\mu g \text{ I/ml. in cuvette}}{\frac{I^{131} \text{ c/s/ml. in cuvette}}{I^{131} \text{ c/s/ml. in urine}}}$$

RESULTS

The results of our procedures and an example of the use of these in our iodine and thyroid studies are in Table I.

TABLE I. RESULTS: AMOUNTS OF STABLE IODINE (I^{127})

		PLASMA (μg/L. AT 6°)	THYROID UPTAKE (μg/KG. FOR 24°)	EXTRATHYROIDAL POOL (μg/KG. 6°)	URINARY EXCRETION (μg/ FOR 24°)
Euthyroid (39 Humans)	Mean	5.99	25.94	2.408	241.2
	S.E.	0.55	2.21	0.701	25.67
	Range	0.86-16.4	6.1-58.9	0.61-6.24	30.2-602
	S.D.	3.44	13.01	1.57	156.2

DISCUSSION

More than 90 per cent of the urinary I^{131} is exchanged in one passage of urine through the AgCl column. Over 98 per cent of the exchanged I^{131} is eluted by Br_2 in 0.05 N H_2SO_4 with no formation of colloidal AgCl.

To check the analytic part of the procedure, we assayed standard solutions of KI after oxidation with Br_2 and $KMnO_4$ and treatment with $NaNO_2$ and urea. Table II shows the results of multiple checks.

TABLE II. ANALYSIS OF KI

CALCULATED I^- (μg/ML.)	DETERMINED I^- (μg/ML.)	ERROR (%)
0.050	0.051	+2.0
0.100	0.098	−2.0
0.150	0.158	+5.0
0.200	0.190	−5.0
0.250	0.251	+4.0

The over-all recovery and analysis of water and urine mixtures of iodide labeled with I^{131} was determined as shown in Table III.

TABLE III. ANALYSIS OF SYNTHETIC MIXTURES

MIXTURE	ADDED (μgI$^-$/ML.)	FOUND (μg/ML.)	ERROR (%)	ERROR (μg/ML.)
Water	None	0.001	–	–
Water	0.050	0.052	+ 1.9	+0.001
Water	0.100	0.105	+ 3.6	+0.004
Water	0.150	0.146	− 3.2	−0.005
Water	0.200	0.194	− 3.7	−0.007
Water	0.250	0.254	+ 1.2	+0.003
Urine	None	0.130	–	–
Urine	0.050	0.200	+10	+0.02
Urine	0.100	0.250	+ 9	+0.02
Urine	0.150	0.280	0	0.0
Urine	0.200	0.300	− 9	−0.03
Urine	0.250	0.400	+ 5	+0.02

The optimum pH for iodine color development was found to be 2.8. Maximum optical density was found at 352 mμ.

The curve of optical density versus concentration of iodide is linear with a slope of 1.7.

This rapid method for determination of urinary iodide employs the concentration of this halide by exchange reaction with AgCl. The AgI^- is treated with Br_2 to elute the iodine as iodate ion, which is then reacted with KI reagent to yield I_2. The iodine color is measured spectrophotometrically. Overall recovery is corrected by radioactivity measurements of added I^{131}. This method is not sufficiently sensitive to measure blood values directly because the levels are so low. The biggest volume which we used was 50 ml. of plasma.

Blank values for all reagents with substitution of urine by water were less than 0.001 μg of iodide per milliliter.

The Barker analysis for iodine is more time-consuming and tedious than ours is, and requires practically an iodine-free work area. We find our method relatively simple and reliable. A single test requires 2 hours, but 4 to 6 tests require no more than 3 hours. This analysis is useful for appraising iodine distribution in the body plasma, thyroid gland uptake, and extrathyroidal pool and space, by employing standard formulas.

SUMMARY

A method is described for concentrating and quantitating urinary iodide. The principle of concentration is the displacement of chloride on a AgCl column by I^-. The iodide is eluted by acidified bromine water; interfering substances are destroyed by an oxidizer ($KMnO_4$); the iodate is converted to iodine by KI or NaI and quantitated spectrophotometrically. Normal values for iodine are given for human plasma, thyroid gland uptake, extrathyroidal pool, and urinary excretion.

REFERENCE

1. Purves, H.: Recovery of Radioactive Iodine From Urine, Nature **169**: 111-112, 1952.

SIMPLIFIED TECHNIQUE FOR DETERMINING SERIAL CHANGES IN PLASMA VOLUME USING I^{131} HUMAN SERUM ALBUMIN

BERNARD ABRAMS, M.D., TILDEN C. EVERSON, M.D., PH.D.,
THEODORE FIELDS, M.S., AND ERVIN KAPLAN, M.D.
CHICAGO, ILL.

IN SOME studies of plasma volume in humans the primary interest may not be the determination of absolute plasma volume values (for which the error of individual determinations is generally stated to be ±5 per cent) but rather the determination of variations in plasma volume. We wish to report a method which may be superior to comparison of absolute plasma volume levels as determined by conventional techniques.

METHOD

Material.—

4 μc I^{131} human serum albumin, HSA.*
Heparin.

Apparatus.—

Scintillation well counter NaI (Th) with use of modified Morton circuit for 300 volt plateau.[2]
Plastic test tubes, ½ inch outside diameter, 4 inches long, with metallic screw cap.†
One 18 gauge intravenous needle.
One 5 or 10 ml. syringe.
One three-way stopcock.
Two heparin-filled glass cannulas.

Procedure.—

1. Twenty-four hours prior to study, the subject is given approximately 4 μc I^{131} HSA intravenously. The volume injected is not of critical importance. (It was elected to take baseline samples after 24 hours since the decline of the plasma level of I^{131} is greatest during the first hours and the escape after the first 24 hours over a period of several hours is relatively small, i.e., less than 1 per cent.)[1]

2. At the time of study an 18 gauge intravenous needle is inserted into a suitable arm vein. The three-way stopcock with attached heparin-filled cannulas is inserted into the needle.

3. At least four 5 ml. samples of blood are withdrawn at timed intervals by substituting a syringe for one of the heparin-filled cannulas. (These initial samples represent the base line—see Fig. 1.)

4. Subsequent 5 ml. samples of blood are withdrawn at desired time intervals during the study period of several hours.

From the Surgical Service and Radioisotope Unit, Veterans Administration Hospital, Hines, Ill., and the Department of Surgery, University of Illinois College of Medicine, Chicago, Ill.

Received for publication Aug. 6, 1956.

*Atomic Energy Commission, Oak Ridge, Tenn., and Abbott Laboratories, North Chicago, Ill.

†Lusteroid Corporation, Maplewood, N. J.

5. Each sample is centrifuged in a test tube at 3,000 r.p.m. for 30 minutes.

6. Two milliliters of plasma are withdrawn from each sample with a pipette and placed in a disposable plastic test tube.

7. Plasma samples are counted in a scintillation well counter for 5 minutes each.

8. After subtraction of background radiation, the number of registers per 5 minutes may be plotted on graph paper. Changes in blood volume are inversely proportional to the registers per 5 minutes above and below the base line (which usually has an imperceptible slope during a several-hour period of study). A typical graph obtained in an experimental study utilizing this method is represented in Fig. 1.

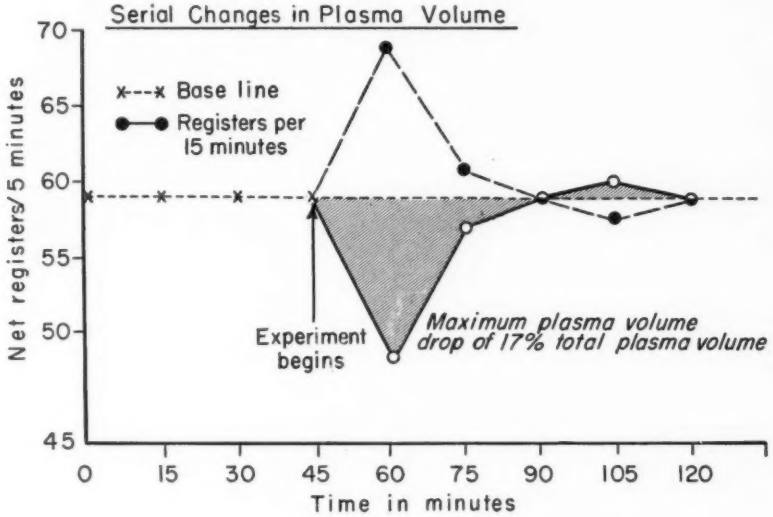


Fig. 1.

VALIDATION OF METHOD

In Table I are listed registers per 5 minutes obtained in four different timed samples in eight normal subjects. As can be seen, the elusion of I^{131} HSA in these subjects was insignificant during the one-hour period of observation, and determinations were reproducible with a high degree of accuracy.

TABLE I. SERIAL DETERMINATIONS (CONTROLS) OF CHANGES IN PLASMA VOLUME (REGISTERS PER 5 MINUTES)

SUBJECT	TIME INTERVAL				RANGE
	15 MIN.	30 MIN.	45 MIN.	60 MIN.	
1	105	104	105	105	104-105
2	167	167	164	165	164-167
3	58	58	58	58	58-58
4	61	60	61	61	60-61
5	54	53	52	52	52-54
6	106	102	105	105	104-105
7	105	104	105	105	104-105
8	91	93	92	91	91-93

SUMMARY

Although the accuracy of individual plasma volume determinations with I^{131} HSA is generally accepted to be ±5 per cent, such a method might not

be sufficiently accurate for studying serial changes in plasma volume because of possible compounding of the 5 per cent error intrinsic in determining absolute plasma volume. A serial method which compares only changes in plasma volume without reference to absolute plasma volume values is described. It is felt that changes in plasma volume can be expressed within an error of 2 per cent by this method.

REFERENCES

1. Sherr, R., and Gerhart, J. B.: Stabilization of Photomultiplier Tubes, Rev. Scient. Instruments **23**: 770-772, 1952.
2. Moir, T. W., Pritchard, W. H., and Ford, A. B.: The Early Disappearance of I^{131} Serum Albumin From the Circulation of Edematous Subjects and Its Implications in the Clinical Determination of the Blood Volume, J. LAB. & CLIN. MED. **47**: 503-512, 1956.

Erratum

In the article by James W. Linman, M.D., and Frank H. Bethell, M.D., entitled "The Plasma Erythropoietic-Stimulating Factor in Man," which appeared in the January issue of the JOURNAL, pages 113-127, the last two numbers in Table VI (page 119) namely, 54.3 and 33.6, should be transposed. On page 125 the second line from the top should end ". . . 260 and 280 mμ."